Free Radical Biology & Medicine 48 (2010) 342–347

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise

Filip J. Larsen [a,b,*], Eddie Weitzberg [a], Jon O. Lundberg [a], Björn Ekblom [a,b]

[a] Department of Physiology and Pharmacology, Swedish School of Sport and Health Sciences, Karolinska Institutet, 11486 Stockholm, Sweden
[b] Åstrand Laboratory of Work Physiology, Swedish School of Sport and Health Sciences, Karolinska Institutet, 11486 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 3 July 2009
Revised 29 October 2009
Accepted 8 November 2009
Available online 12 November 2009

Keywords:
VO_{2max}
Exercise
Dietary nitrate
Nitrite
Nitric oxide
Performance
Postexercise
Hypotension
cGMP
Free radicals

ABSTRACT

The anion nitrate—abundant in our diet—has recently emerged as a major pool of nitric oxide (NO) synthase-independent NO production. Nitrate is reduced stepwise in vivo to nitrite and then NO and possibly other bioactive nitrogen oxides. This reductive pathway is enhanced during low oxygen tension and acidosis. A recent study shows a reduction in oxygen consumption during submaximal exercise attributable to dietary nitrate. We went on to study the effects of dietary nitrate on various physiological and biochemical parameters during maximal exercise. Nine healthy, nonsmoking volunteers (age 30 ± 2.3 years, VO_{2max} 3.72 ± 0.33 L/min) participated in this study, which had a randomized, double-blind crossover design. Subjects received dietary supplementation with sodium nitrate (0.1 mmol/kg/day) or placebo (NaCl) for 2 days before the test. This dose corresponds to the amount found in 100–300 g of a nitrate-rich vegetable such as spinach or beetroot. The maximal exercise tests consisted of an incremental exercise to exhaustion with combined arm and leg cranking on two separate ergometers. Dietary nitrate reduced VO_{2max} from 3.72 ± 0.33 to 3.62 ± 0.31 L/min, $P<0.05$. Despite the reduction in VO_{2max} the time to exhaustion trended to an increase after nitrate supplementation (524 ± 31 vs 563 ± 30 s, $P=0.13$). There was a correlation between the change in time to exhaustion and the change in VO_{2max} ($R^2=0.47$, $P=0.04$). A moderate dietary dose of nitrate significantly reduces VO_{2max} during maximal exercise using a large active muscle mass. This reduction occurred with a trend toward increased time to exhaustion implying that two separate mechanisms are involved: one that reduces VO_{2max} and another that improves the energetic function of the working muscles.

Dietary sources of nitrate and nitrite have recently emerged as major pools of NO synthase-independent NO production under certain conditions [1–3]. This is an alternative to the classical pathway in which NO is produced by oxidation of L-arginine in a reaction catalyzed by the NO synthases. Plasma levels of nitrate and nitrite are dependent on our diet but also on oxidation of NO produced by NO synthases (NOSs). Dietary nitrate is mainly found in green leafy vegetables, whereas nitrite is a common preservative in cured meat products [4,5].

After ingestion nitrate is rapidly absorbed in the upper gastrointestinal tract and the bioavailability is 100%. For yet unknown reasons circulating nitrate is actively extracted by the salivary glands and secreted in saliva. In the mouth nitrate is partly converted to nitrite by nitrate-reducing commensal bacteria. Swallowed nitrite can then enter the systemic circulation thereby creating an enterosalivary circulation of these anions. Indeed, a recent study shows that ingestion of nitrate results in a sustained increase in circulating nitrite levels [6]. Nitrite can be further reduced in blood and tissues to form bioactive NO. The clearest evidence of an NO-like bioactivity from dietary nitrate in humans is the reduction in blood pressure seen after ingestion of this anion [7–9]. There are several enzymatic and nonenzymatic pathways for the one-electron reduction of nitrite to NO. These include deoxyhemoglobin [10], deoxymyoglobin [11], xanthine oxidase [12], and enzymes of the mitochondrial respiratory chain [13]. Nitrite-derived NO has been suggested to possess several physiological functions such as vasodilation [10,14] and protection against ischemia–reperfusion injury [15,16]. These effects are especially pronounced during increased metabolic demand and hypoxic stress, i.e., ischemia–reperfusion injury (for review see [2,17]). Tissue acidosis and low oxygen tension are present during physical exercise and in this metabolic state reduction of nitrite is probably enhanced. Earlier studies have shown that inhibition of NOS-derived NO increases oxygen consumption in vivo in dogs [18] and in rats [19]. Mitochondrial studies also reveal that the efficiency of oxidative phosphorylation becomes more efficient in the presence of NO [20].

In a recent study we showed that dietary nitrate not only reduces resting blood pressure but also affects vital physiological parameters during exercise [8]. Specifically, we found that nitrate ingestion resulted in a significant reduction of VO_2 on submaximal work rates. This highly surprising effect occurred without accumulation of lactate,

* Corresponding author.
E-mail address: filip.larsen@ki.se (F.J. Larsen).

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doi:10.1016/j.freeradbiomed.2009.11.006

indicating that energy production had become more efficient. The mechanism is still not known but we speculated that NO or a closely related species in some way reduces proton leakage over the mitochondrial membrane. In the cited study nitrate reduced VO_2 at submaximal workloads but on maximal work rates this did not reach significance.

In this study we wanted to specifically investigate the effect of nitrate supplementation on maximal combined arm and leg exercise. The metabolic and circulatory response to maximal exercise is quite different compared to the submaximal response. At very high work rates the body has reached its maximum capacity to consume oxygen (VO_{2max}) and increases no further despite increasing workload. The VO_{2max} is largely determined by the maximal cardiac output and especially maximal stroke volume [21,22]. Indeed, to achieve a true VO_{2max} the active muscle mass needs to be at least 50% of the total muscle mass [23]. The oxygen cost of submaximal exercise, on the other hand, is largely determined by the efficiency of the contracting muscles. In this study, we sought to investigate whether dietary nitrate would also influence VO_{2max} during exercise with large active muscle groups and if this would have any effect on the time to exhaustion during incremental exercise. We also studied the effects of nitrate supplementation on plasma nitrite kinetics during exercise and blood pressure in the acute recovery period after this exhaustive exercise.

Materials and methods

Subjects and exercise protocol

Nine (seven male, two female) healthy, nonsmoking volunteers (age 30 ± 2.3 years, VO_{2max} 3.72 ± 0.33 L/min) participated in this study. All subjects were informed about the experimental procedures and gave their written consent. The protocol was approved by the regional ethics committee in Stockholm.

The maximal exercise tests were performed using two combined electronically braked arm and leg ergometer cycles (Monark 839E; Monark, Vansbro, Sweden). One of these was used as an ordinary leg cycle ergometer, whereas the other was placed on an aluminum frame and used as an arm ergometer, with handgrips mounted on the cranks. The subjects were seated behind the arm ergometer, with the height of the saddle adjusted so that the arms, when extended, were level with the heart.

The ratio of arm to leg work in all tests ranged between 20:80% and 25:75% of the total rate of work. The cadence was set to 80 rpm for both arms and legs. The cadence figures, one for legs and the other for arms, were available on two computer screens in front of the subject as previously reported [24].

Heart rate (HR) was recorded by a heart-rate monitor (Model Polar S610; Polar Electro OY, Finland). Oxygen uptake (VO_2) and pulmonary ventilation (VE) were determined by the Douglas bag method or with an online gas analyzer system (AMIS 2001; Innovision A/S, Odense, Denmark). These two methods have been validated against each other [25] and the two individual setups were also validated against each other before the commencement of the tests. The gas analyzers were calibrated with high-precision calibration gases ($16.00 \pm 0.01\%$ O_2 and $4.00 \pm 0.01\%$ CO_2; Air Liquide, Kungsängen, Sweden). Before each test, ambient conditions were measured and the gas analyzers and inspiratory flow meter were calibrated. A 6–20 RPE scale was employed to monitor the subject's rating of perceived exertion (RPE) [26].

The VO_{2max} was recorded as the highest average oxygen uptake maintained during at least 45 s. The criteria for a true VO_{2max} were the attainment of a plateau in oxygen uptake (increase less than 150 ml/min during the last minute of exercise) despite an increase in workload and at least one of the following [27]: a respiratory exchange ratio (RER) above 1.1, above 8 mM blood lactate, or an RPE of at least 18. The maximal effort test continued until volitional exhaustion or was terminated when the cadence decreased below 70 rpm. All subjects were familiarized with the testing procedure and allowed to train at the combined arm and leg ergometer at an initial occasion. When the subjects felt confident using the ergometer and were able to synchronize the arm and leg cranking, the practice session was terminated. During this first session the workload for the subsequent sessions was determined with respect to achieved workload, heart rate, and perceived exertion.

In a separate set of experiments we investigated the acute metabolic response to a single dose of sodium nitrate. Seven healthy nonsmoking male subjects (mean age 39 years) cycled at a very light workload (86 ± 4 W) until steady-state oxygen consumption was attained (approximately 5 min). Gas exchange was measured as during the maximal exercise test. After completion of the first test, a single dose of 0.033 mmol $NaNO_3$/kg body wt was consumed and 60 min later the subject repeated the test at the same workload and cadence. As a reference test the seven subjects repeated the test on a separate day but with ingestion of placebo (NaCl) instead of nitrate after the first exercise bout. These subjects were not fasted and did not adhere to the nitrate-free diet described below. No blood samples were taken in these subjects.

Dietary manipulations

After the familiarization session, the subjects were tested on two occasions. All tests were performed after an overnight fast. This study was randomized and double-blinded with a crossover design. The washout period between the two trials was at least 7 days. The following two dietary supplementation protocols were used.

(1) Nitrate supplementation: the subjects were told to consume a diet with low nitrate content (i.e. avoid all vegetables, tea, nicotine, some fruits, cured meats, and fish) for 2 days before the test. The subjects received 0.033 mmol $NaNO_3$/kg body wt three times daily. The last dose was taken 40 min before the exercise test was initiated.

(2) The placebo protocol was the same as in (1) but the subjects received an equimolar amount of NaCl (placebo) instead of nitrate. Initially 10 subjects were recruited but 1 subject admitted not being able to adhere to the dietary restrictions and was excluded from the study.

Table 1
Metabolic and circulatory responses to maximal exercise in nine individuals after dietary supplementation with nitrate or placebo

	VO_{2max} (L/min)	VE_{max} (L/min)	HR_{max} (bpm)	RER	O_2 pulse (ml/beat)	VE/VO_2	TTE (s)	La_{max} (mM)
Nitrate	3.62 ± 0.31	141 ± 6	183 ± 3	1.10 ± 0.01	19.8 ± 1.7	26.5 ± 1.5	563 ± 30	10.97 ± 0.45
Placebo	$3.72 \pm 0.33^{*}$	140 ± 9	183 ± 4	1.09 ± 0.02	$20.5 \pm 1.9^{**}$	$25.4 \pm 1.4^{**}$	524 ± 31	10.46 ± 0.50

VO_{2max}, maximal oxygen consumption; VE_{max}, maximal pulmonary ventilation; HR_{max}, maximal heart rate; RER, respiratory exchange ratio (i.e., maximal oxygen consumption/maximal rate of exhaled carbon dioxide); O_2 pulse, milliliters of oxygen consumed per heart beat; VE/VO_2, liters of pulmonary ventilation per liter of consumed oxygen; TTE, time to exhaustion during the maximal work test; La_{max}, maximal blood lactate at end of exercise.

* $P<0.05$, nitrate vs placebo.

** Trend, $P=0.05$–0.1, nitrate vs placebo.

Blood sampling

In brief, a small catheter was inserted into an antecubital vein for venous blood sampling. Venous blood was drawn with a 5-ml syringe and mixed with nitrite-free EDTA in a 10-ml test tube and immediately centrifuged at 3000 g for 10 min at 4°C. The plasma was then separated and kept at −80°C. Blood was drawn at rest, after warm-up, and at 1, 8, 20, and 30 min postexercise. The plasma concentrations of nitrate and nitrite were analyzed by a chemiluminescence assay as described previously [6]. Capillary blood samples (20 μl) were collected from the fingertip and analyzed for lactate using a Biosen C-Line sport analyzer (EKF Diagnostics, Magdeburg, Germany).

Blood pressure measurement

The blood pressure was measured by the same investigator, on the subject's same arm each time, with a stethoscope and an inflatable sphygmomanometer placed 1–2 cm above the elbow. The size of the cuff was chosen according to the circumference of the subject's upper arm. The first Korotkoff sound was interpreted as systolic and the fifth phase as diastolic pressure. Blood pressure measurements were made with the subject lying in the supine position. The resting blood pressure was measured twice after the subject had been resting in a quiet room for at least 30 min.

Statistics

Results are expressed as means ± standard error of the mean. Paired *t* test was used to evaluate the difference between the placebo and the nitrate groups where appropriate. A Bonferroni correction was applied when multiple comparisons were done outside of the original hypothesis. To investigate the influence of time and treatment (placebo or nitrate), the data were treated with a two-way analysis of variance (ANOVA) with repeated measures on both time and treatment. The data were assessed to determine normal distribution, and post hoc analyses were undertaken using Tukey's HSD. Postexercise measures of blood pressure were expressed as delta changes from the preexercise values and analyzed for differences over time with one-way ANOVA. The significance level was set at $P<0.05$. However, *P* values between 0.05 and 0.15 were also considered to be suggestive of null hypothesis rejection and are included in the results as statistical trends.

Results

Circulatory responses to maximal exercise

After nitrate supplementation VO_{2max} was reduced from 3.72 ± 0.33 to 3.62 ± 0.31 L/min ($P<0.05$, Table 1). A plot of each subject's individual response to nitrate supplementation can be seen in Fig. 1. Heart rate and pulmonary ventilation were unaffected by nitrate supplementation. Time to exhaustion showed a near-significant increase after nitrate supplementation ($P=0.13$). There was a positive correlation between the change in VO_{2max} and the change in time to exhaustion ($P=0.042$, $R^2=0.467$, Fig. 2). There was no change in lactate formation (Fig. 3) during or after maximal exercise.

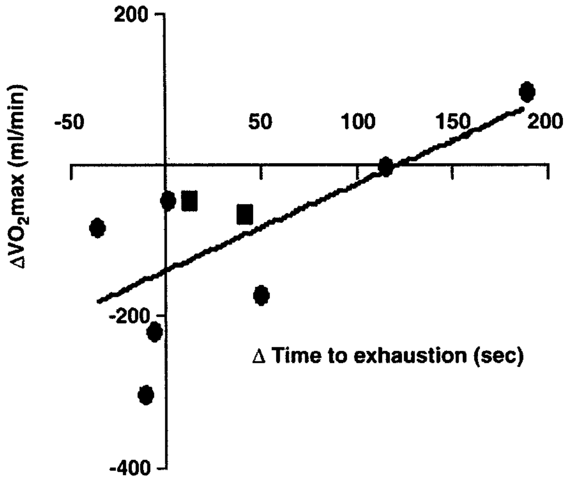

Fig. 2. Graph showing the correlation between the difference in VO_{2max} and the difference in time to exhaustion between two occasions when nine subjects ingested either nitrate ($NaNO_3$) or placebo (NaCl) for 3 days before the experiment. $P<0.05$, $R^2=0.47$.

Submaximal response to a single dose of nitrate

Sixty minutes after consumption of a single dose of nitrate VO_2 decreased from 1.45 ± 0.08 to 1.37 ± 0.09 L/min ($P<0.05$) but was unchanged in the placebo trials (individual responses in Fig. 4). HR, VE, and RER were unaffected by the supplementation.

Nitrate and nitrite kinetics

Resting plasma concentrations of nitrate were higher after nitrate supplementation (230 ± 31 μM) compared to placebo (17.3 ± 3.0 μM, $P<0.001$). The exercise bout did not affect the nitrate concentrations at any time point after termination of the exercise. Conversion of nitrate to nitrite was evident from the increased plasma nitrite levels after the supplementation period (142 ± 35 nM) compared to placebo (61 ± 11 nM, $P<0.01$, ANOVA, Fig. 5). There was a tendency for the nitrite levels to decrease in the placebo group at 1 min after exercise compared to the resting levels ($P=0.06$, Fig. 5).

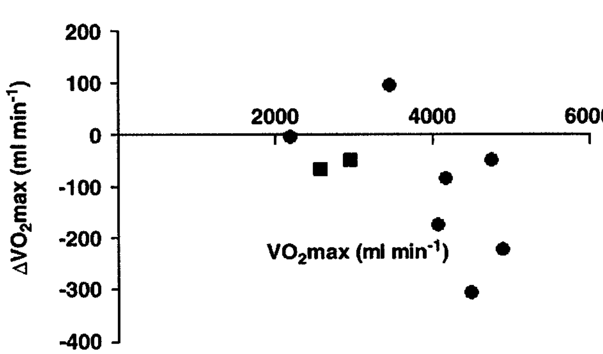

Fig. 1. The individual changes in VO_{2max} after dietary nitrate supplementation compared to placebo. The values on the x axis represent the VO_{2max} during the placebo trial. Filled circles, male subjects; filled squares, female subjects.

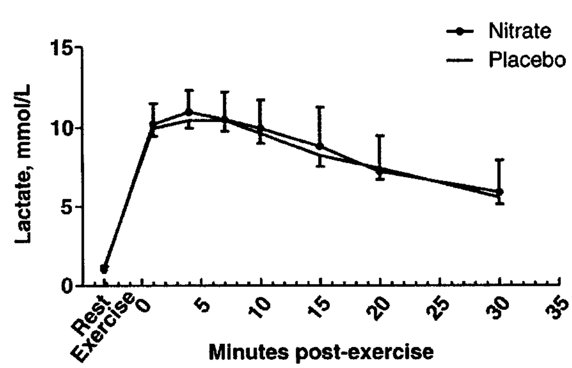

Fig. 3. Plasma lactate levels in nine subjects performing a maximal exercise test after 3 days of dietary supplementation with sodium nitrate or placebo.

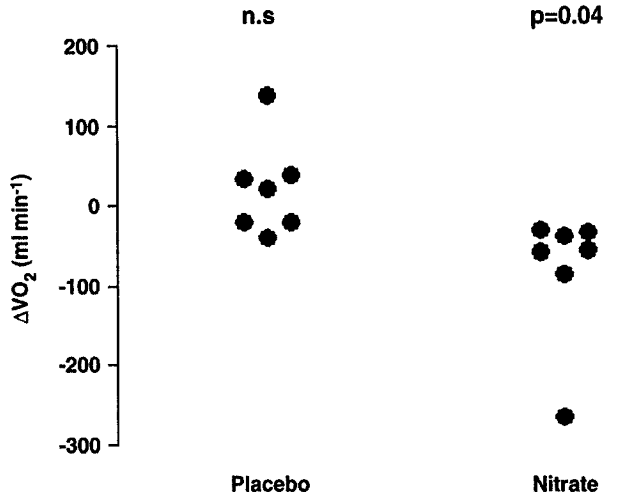

Fig. 4. Acute effects of dietary nitrate on VO_2 during light exercise. Seven subjects performed cycling (86 ± 4 W) on an ergometer until steady-state oxygen consumption was achieved. Immediately after exercise the subjects consumed a single dose of 0.033 mol/kg body wt of either sodium nitrate or placebo. 60 min later the cycling was performed again at the same work rate and cadence. All seven subjects did both the placebo and the nitrate trials. The values presented are the differences between the first cycling bout and that after nitrate or placebo.

Blood pressure response

There was no significant change in resting blood pressure (nitrate, systolic 109 ± 3, diastolic 69 ± 2 mm Hg; and placebo, systolic 109 ± 5, diastolic 70 ± 2 mm Hg). However, at 2 min after the maximal exercise, the diastolic blood pressure decreased significantly compared to the resting blood pressure in the nitrate group, but not in the placebo group (Fig. 6).

Cyclic guanosine monophosphate (cGMP)

The plasma concentrations of cGMP increased in both groups after exercise compared to rest ($P<0.001$, Fig. 7) and were elevated for the entire 30 min of the investigation period. There were no differences between nitrate supplementation and placebo in absolute values or relative increases in cGMP.

Renin–aldosterone response to nitrate supplementation and exercise

There was no significant change in plasma renin concentration between nitrate (5.5 ± 1.2 nM) and placebo (7.1 ± 0.8 nM, $P=0.11$) at rest nor after exercise (36.3 ± 6.1 and 31.5 ± 7.2 nM, $P=0.46$).

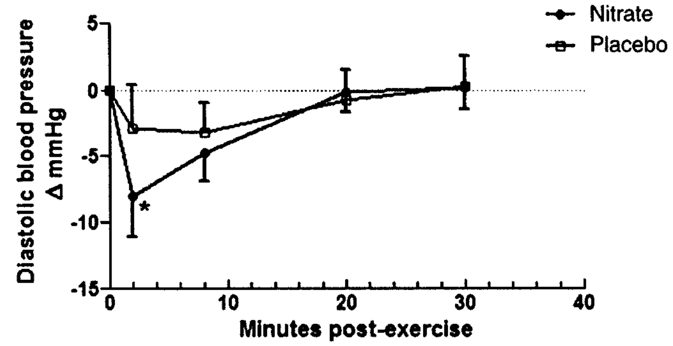

Fig. 6. Change in diastolic blood pressure at rest and 2, 8, 20, and 30 min after maximal exercise in nine subjects after 3 days of dietary supplementation with sodium nitrate or placebo. $^*P<0.05$ for difference from resting blood pressure (ANOVA).

Neither were there any differences in plasma aldosterone concentrations at rest (nitrate, 233 ± 47; placebo, 193 ± 43 pM; $P=0.39$) or after exercise (nitrate, 384 ± 53; placebo, 332 ± 76 pM; $P=0.23$).

Plasma levels of amino acids

To verify that there was no difference in the intake of dietary amino acids preceding the two trials, we analyzed plasma levels of 20 amino acids, including L-arginine and citrulline. There were no differences in any of the amino acids analyzed (data not shown).

Discussion

This study highlights several important aspects of dietary nitrate supplementation regarding circulatory and metabolic parameters during and immediately after exercise. Nitrate supplementation seems to give a lower VO_{2max} compared to a low-nitrate diet. This change occurred without any effect on VE_{max}, HR_{max}, RER, or maximal lactate values. These data emphasize the physiological importance of our findings because modest amounts of nitrate, which can easily be consumed in a vegetable-rich diet, provided the effects. A highly surprising observation is the trend toward increased time to exhaustion after nitrate supplementation that occurred despite a reduced VO_{2max}. Normally, a reduction in VO_{2max} is very tightly coupled to decreases in work performance [28], as classically seen with hypoxic gas inhalation [29,30]. One could argue that this unexpected effect was due to a greater anaerobic energy contribution but we found no change in lactate formation, thereby ruling out this possibility. However, far from all oxygen-consuming processes are coupled to proton pumping and ATP synthesis. Ion

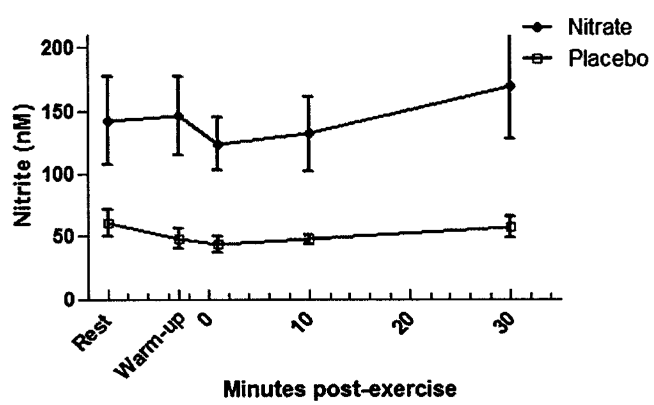

Fig. 5. Plasma nitrite levels at rest in nine subjects during warm-up and up to 30 min after maximal exercise on two occasions: after 3 days of dietary supplementation with sodium nitrate or placebo.

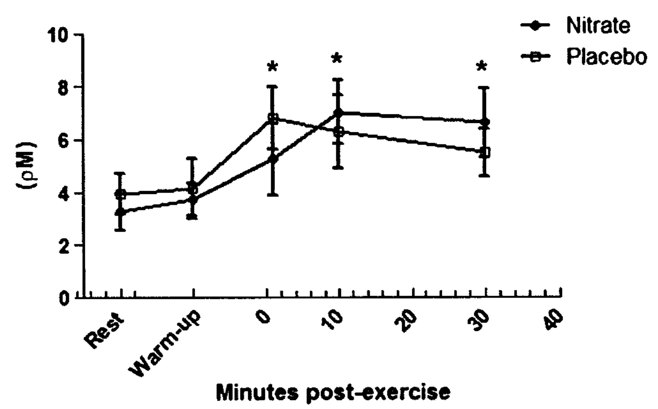

Fig. 7. Plasma levels of cGMP at rest, during warm-up, and up to 30 min after maximal exercise on two occasions: after 3 days of dietary supplementation with sodium nitrate or placebo.

channel leaks, proton leakage, cellular transport of nutrients, heat production, etc., all consume oxygen. An explanation for our finding could be that the decrease in VO_{2max} is coupled to a decrease in one of these non-ATP-producing but O_2-consuming functions in muscle tissue. Indeed, several recent reports have found an improved efficiency of the mitochondrial oxygen consumption in vitro when adding NO donors [31] and a decreased efficiency when adding the NOS inhibitor L-NAME in vivo [18]. Another possibility that has previously been investigated [32] is that an increased NO availability reduces the oxygen-consuming constriction of the blood vessel wall. However, this reduced O_2 consumption would not be able to reduce VO_{2max} if the cardiac output is the limiting factor for the oxygen transport. It is possible that there are two independent mechanisms involved: one that lowers VO_{2max}, and this has a slight negative impact on the time to exhaustion. The other mechanism, however, improves performance speculatively through an improved muscular efficiency that we found in our earlier study [8]. Yet another explanation could be related to the mitochondrial membrane potential, which correlates with proton leakage, so that mitochondria with lower membrane potential are more efficient energy converters [33]. Proton leak is defined as non-ATP-producing proton cycling over the inner mitochondrial membrane and accounts for 15% of active and 20% of resting oxygen consumption [34]. NO has been proposed to extend the oxygen gradient in tissues [35] by partial inhibition of mitochondria close to the feeding artery. In this situation oxygen will distribute over a larger population of mitochondria, lowering the membrane potential and theoretically also reducing proton leak by the same mechanism.

In a study by Rassaf and colleagues using perfused rat hearts, a decrease in left ventricular developed pressure, decreased contractile function, and lower oxygen consumption were observed when nitrite was added in the perfusate [36]. If the lower VO_{2max} originates from an effect on the central circulatory organs such as a smaller stroke volume or decreased contractile capacity of the heart, it is highly likely that we would have found a shortened time to exhaustion during the maximal exercise test. More importantly, we did not see any effect on maximal heart rate, implying no effect on the inotropy of the heart.

In this study we found that a single dose of nitrate reduced VO_2 by 80 ml/min. This is somewhat smaller than the effect on VO_{2max} (100 ml/min) and only about half of what we reported in an earlier study [8]. These different responses to nitrate might be related to the very light workload in the acute experiments or the fact that the subjects were not fasting or consuming a nitrate-free diet before the experiment. Nevertheless, this implies that there is an acute effect of nitrate supplementation that can be further augmented by a longer supplementation period.

The results of this study are interesting in the context of research done on high-altitude-living Tibetans. This group of people has adapted to high altitude and low barometric pressure through several generations. Tibetans living at 4400 m above sea level (asl) have significantly lower VO_2 at submaximal work rates and similar [37] or lower [38] VO_{2max} but higher maximal work rates compared to inhabitants of lower altitudes. Interestingly, another recent study shows that Tibetans living at 4200 m asl have >10-fold higher circulating nitrate and nitrite levels than U.S. residents at sea level [39]. It is tempting to speculate that the Tibetans' adaption to the lower barometric pressure at least partly is mediated by increased circulating levels of nitrate and nitrite, probably through the upregulation and/or activation of eNOS.

We also found that plasma nitrite seemed to be consumed in the placebo group during exercise ($P = 0.06$ rest vs 1 min postexercise) and recovered to preexercise levels after 30 min of rest. We interpret the consumption of plasma nitrite as a faster rate of nitrite-to-NO reduction than the rate of oxidation of NO back to nitrite. This seems to contradict earlier findings that suggest an increase in plasma nitrite after exercise [40,41]. The time aspect is probably important here; the acute effect of exercise might be a consumption of nitrite but at the same time the increased shear stress on the endothelium might increase the enzymatic formation of NO through activation of eNOS, thereby elevating the nitrite levels for hours after exercise. The increased levels of circulating cGMP indicate an increased NO production but do not distinguish nitrate-derived NO from NO produced by NOS.

We also studied the effects on blood pressure before and after exercise. In previous larger studies we [7] and others [42] have demonstrated a significant reduction in resting diastolic blood pressure. Others have seen a rather dramatic acute effect on both systolic and diastolic blood pressure after consumption of nitrate-rich beetroot juice [9]. In the present study we did not find any significant reduction in resting blood pressure, probably because of the weaker statistic power of this study, the lower dose, or the shorter supplementation period (2 vs 3 days). In addition, Webb and colleagues [9] found that the maximum blood pressure reduction from nitrate occurred 3 h after ingestion, whereas in the present study blood pressure was measured only 40 min after the last ingestion.

When exercise is terminated, there is a temporary decrease in blood pressure, below resting values, as blood remains pooled in the dilated muscle beds (for review see [43]). The mechanism of signaling molecules involved in this postexercise hypotension remains unclear, but NO [44], adenosine [45], and atrial naturetic peptide [43] have all been suggested as possible mediators. In this study, the diastolic blood pressure was significantly lower than baseline (−7 mm Hg, $P = 0.04$) in the nitrate group at 2 min after the termination of exercise. There was no significant reduction in blood pressure in the placebo group over the investigation period. Studies using NO synthase inhibitors in humans have shown a suppressed hypotensive response after a bout of resistance exercise [44]. NO produced during exercise has been related to the activation of eNOS due to increased shear stress on the endothelium. This study indicates that nitrite-derived nitric oxide is also important in the postexercise hypotensive response. Inhibition of nitric oxide synthesis increases blood pressure and has also been shown to increase the aldosterone levels in humans. Therefore we investigated whether the blood pressure effects in this study originated from alterations in the aldosterone or renin system as has previously been shown with NO inhibitors [46]. We found no effect on plasma levels of aldosterone or renin at rest or after exercise with nitrate supplementation compared to placebo. The mechanism behind the effect on blood pressure remains unclear, but others have shown an increased hypoxic vasodilation and increased blood flow [10] with nitrite infusions. It is more likely that this nitrite-induced vasodilation is responsible for the effects on blood pressure, rather than alterations in blood pressure-regulating hormones. Alternatively, nitrite may be converted to vasodilatory NO in the vessel wall, possibly via intermediate formation of a nitrosothiol.

In summary, our results indicate that nitrate supplementation decreases VO_{2max} at maximal combined arm and leg exercise. Surprisingly, this effect occurred with a trend toward increased performance. There is a great interest in the effects of our diet in sports medicine and our findings add nitrate to the list of nutrients that may be of importance in this field of research.

Acknowledgments

This work was supported by grants from the Swedish Research Council, Vinnova (CIDaT), the Torsten and Ragnar Söderbergs Foundation, the European Union's 6th Framework Programme (Eicosanox), and the Swedish Heart–Lung Foundation.

Note added in proof. Consistent with the present findings, during the review of the manuscript, Bailey et al. demonstrated in healthy volunteers that dietary nitrate (in the form of beetroot juice) reduced

the O_2 cost of submaximal exercise and also showed an enhanced tolerance to high-intensity exercise [47].

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QUARTERLY REPORT TO THE EDITOR ON TOPICS OF CURRENT INTEREST

NITRATES AND NITRITES IN MEAT PRODUCTS

The use of sodium and potassium nitrate as curing agents for meats has been an established practice for so long that its origin is unknown. The nitrates have no more preservative action than common salt (1), but do have a special function. The function in the curing process is to fix and heat-stabilize the red color in meat. This color fixation takes place through the reduction of nitrate to nitrite by bacterial action and the reaction of the nitrite with hemoglobin to form nitric oxide hemoglobin. Heat will convert the nitric oxide hemoglobin to nitric oxide hemochromogen, which is also a red pigment. Thus, both pigments may be present in cured meats.

Since nitrate must be changed to nitrite by bacterial action before the above reaction can take place, unsatisfactory or insufficient formation of nitrite results in insufficient or irregular color fixation. It was found that a combination of nitrate and nitrite in the curing salt or pickling solution gave the most satisfactory results (1). Combinations of potassium or sodium nitrate and nitrite are permitted as curing ingredients for meat prepared under Federal meat inspection with the limitations of no more than 200 parts per million of nitrite in the finished product (2).

NATURAL OCCURRENCE OF NITRATE IN FOOD

The normal urinary output of nitrate averages 0.5 gram per day primarily derived from vegetables in the diet. It has been shown that the nitrate content of green vegetables varies from 3,636 p.p.m. for spinach to 50 p.p.m. for asparagus. Strained baby foods were found to contain from 0 p.p.m. for squash and tomatoes to 833 p.p.m. for spinach (3). Traces of nitrate are also found in many potable water supplies. There have been no reports that nitrite occurs in plants, although it has been demonstrated that human blood contains 10 micrograms per 100 cc. as an average (0.1 p.p.m.) (4)

CHRONIC TOXICITY OF NITRATE AND NITRITE

Nitrate. Sodium nitrate has been fed to rats at levels up to 10% in the diet for their life-time (5). Other than some depression in growth at levels above 1% of nitrate, no adverse effects were noted in these animals.

Two dogs were fed 2% sodium nitrate in their diet for a period of 105 and 125 days respectively. No adverse effects were noted and no disturbance in the blood was observed in these animals. Gross and microscopic pathological study of the rats showed nothing attributable to the nitrate

Lehman, A. J. (1958) Quarterly reports to the editor on topics of current interest - Nitrates and nitrites in meat products. In: Association of Food and Drug Officials. 22:136-138.

except changes of inanition at 10%, with no morphological difference from the controls at 5% or less in the diet. Nothing of significance was observed in the dogs.

Nitrite. Rats were fed a sodium nitrite supplement for a period up to 168 days (6). In a typical experiment one rat received a total of 0.167 gram sodium nitrite in 121 days. This represents about 93 p.p.m. in the daily diet. No effects on growth and organ weights of important organs were noted.

In a similar experiment in cats, one animal received about 4.1 grams total dose of sodium nitrite during a period of 105 days. This represents approximately 390 p.p.m. nitrite in the daily diet. Again, no effects on growth rate and weight of important organs were noted.

No histopathological examination was done on any of the animals fed nitrite.

THE EFFECT OF SODIUM NITRITE ON BLOOD PIGMENTS

The reaction of nitrite on the hemoglobin of meat has already been mentioned. In the body the usual effect of nitrite is on the hemoglobin of the blood. There is some controversy concerning the molar ratios involved in the interaction of nitrite with hemoglobin to produce methemoglobin (7). The ratios are stated to vary from 2 mols of nitrite to 1 mol of hemoglobin to 0.5 to 1. Thus, 1 gram of sodium nitrite could convert from 463 to 1855 grams of hemoglobin to methemoglobin.

The rate of conversion of methemoglobin to hemoglobin is also of great importance. From the evidence presently available this conversion proceeds at a rate approximating 10–15% of the total methemoglobin in the blood per hour. Thus, the body mechanisms are capable of rapidly reducing methemoglobin to the oxygen-carrying hemoglobin. It is of interest to note that the normal blood of man contains about 0.7% methemoglobin.

THE HAZARD OF RESIDUAL NITRITE IN MEAT

An individual consuming 100 grams of corned beef containing 200 p.p.m. residual nitrite would ingest 20 milligrams of nitrite. This represents about 13 p.p.m. in the day's diet. In light of the facts related above, this quantity of nitrite, if all absorbed, could convert approximately 10–40 grams of hemoglobin to methemoglobin representing about 1.4 to 5.7 per cent of the total blood pigment. In view of the fact that a concentration of 40% methemoglobin in the blood is necessary before compensatory mechanisms of the body are brought into play to increase the oxygen supply to the tissues, the methemoglobin contributed by 20 milligrams of ingested sodium nitrite is of no significance.

It must be borne in mind that the most significant feature in estimating

the health hazards of nitrite is the molar ratio between the nitrite utilized and the methemoglobin formed. As already pointed out 1 gram of sodium nitrite could convert from 463 to 1855 grams of hemoglobin to methemoglobin. The blood of the average adult contains about 700 grams of hemoglobin. As stated previously a concentration of about 40 per cent methemoglobin in the blood brings about compensatory mechanisms to maintain the oxygen supply to the tissues. A concentration of 80–85 per cent is considered lethal (7). Because of the small molecular weight of sodium nitrite, 69, in relation to the large molecular weight of hemoglobin, 64,000, only a small margin of safety exists between the amount of nitrite that is safe and that which may be dangerous. The margin of safety is even more reduced when the smaller blood volume and the corresponding smaller quantity of hemoglobin in children is taken into account. This has been emphasized in the recent cases of nitrite poisoning in children who consumed weiners and bologna containing nitrite greatly in excess of the 200 p.p.m. permitted by Federal regulations (8). The application of nitrite to other foods is not to be encouraged.

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A. J. L.

Brief Communication: Feeding Studies of Nitrilotriacetic Acid and Derivatives in Rats [1,2]

W. Lijinsky,[3] M. Greenblatt,[4] *and* C. Kommineni,[5,6] *Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska 68105*

SUMMARY—Groups of 15 male and 15 female MRC rats were given 0.5% nitrilotriacetic acid (NTA) or 0.5% iminodiacetic acid (IDA) in drinking water, with and without 0.2% sodium nitrite ($NaNO_2$) or 0.5% sodium nitrate ($NaNO_3$) for most of their lifespans. The dose was 20 ml solution per animal 5 days a week. The expected in vivo nitrosation product, nitrosoiminodiacetic acid (NIDA) was administered similarly at 0.2% in drinking water. The total dose received by each animal was 42 g NTA, IDA, or $NaNO_3$, 16 g $NaNO_2$, or 14.4 g NIDA. No significant difference in tumor induction was noted between the treated groups or between them and the untreated controls.—J Natl Cancer Inst 50: 1061–1063, 1973.,

NITRILOTRIACETIC ACID (NTA) has been considered a possible replacement for "phosphate" as a component of household detergents, and it seemed probable several years ago that NTA would be used on a large scale. When a foreign compound can rapidly be widely disseminated, it is desirable to test the safety of the material (such as NTA) by chronic administration to animals. Because amines like NTA could react with nitrite in the stomach or other media to form *N*-nitroso compounds (*1*, *2*), many of which are carcinogenic (*3*), we designed our test of NTA to examine this possibility also. NTA, although a tertiary amine, could contain the corresponding secondary amine, iminodiacetic acid (IDA), as impurity. This compound forms the *N*-nitroso derivative nitrosoiminodiacetic acid (NIDA) by reaction with nitrous gases (*4*); the same reaction occurs with nitrites in dilute acid solution. NTA could also react with nitrite in weakly acid solution to form NIDA (*5*); this reaction proceeds at a reasonable rate, even at body temperature. The reduction of nitrate to nitrite by bacteria in the stomach has been suggested as a source of nitrosamines when food containing amines is ingested (*6*). As a test of this possibility, iminodiacetic acid was administered with sodium nitrate ($NaNO_3$). The tests were completed with NIDA itself.

MATERIALS AND METHODS

Chemicals.—NTA and IDA, obtained as the disodium salts from Aldrich Chemical Co. (Milwaukee, Wis.), were used without purification. Sodium nitrite ($NaNO_2$) and $NaNO_3$ were from Fisher Scientific Co. (Silver Spring, Md.).

NIDA was prepared by dissolving 50 g sodium iminodiacetate in 100 ml water; 100 g of ice plus 100 ml concentrated HCl were added. The solution was cooled in ice, and 35 g $NaNO_2$ was added slowly during 30 minutes. The solution was allowed to stand in ice for a half hour, then was evaporated to 100 ml with a rotary evaporator (60° C bath temperature) and filtered. The filtrate was extracted 3 times with 500–600 ml ether, and the combined ether extracts were dried with anhydrous magnesium sulfate. The ether solution was distilled on a warm-water bath until about 150 ml remained; this was further evaporated to about 30 ml in a stream of nitrogen at room temperature. The crystalline solid was filtered off, washed with a little ether, and allowed to dry in air. The yield was 28 g (70%) of almost colorless crystals, mp 145.5–146.5° C.

The mass spectrum (70 eV) showed a parent ion at m/e 162 corresponding to $C_4H_6N_2O_5$, with fragments at m/e 132 (loss of NO), and 117 (loss of COOH).

Animals and treatments.—Male and female MRC rats, originally obtained from the Medical Research Council, Carshalton, England, and subsequently bred in our laboratory, were grouped 5 to a polycarbonate cage on

1 Received January 16, 1973; accepted January 24, 1973.

2 Supported by Public Health Service contract 43-68-959 from the National Cancer Institute.

3 *Address for inquiries:* The Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830.

4 *Present Address:* National Cancer Research Laboratories, University of Otago Medical School, Dunedin, New Zealand.

5 *Present address:* Food and Drug Administration, Washington, D.C. 20024.

6 We acknowledge the technical assistance of Mrs. Donna K. Nelson and Mrs. Linda Banzet.

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Sanicel bedding and fed Rockland diet in pellets. The animals were 8–10 weeks old at the beginning of treatment.

Solutions for feeding were prepared by dissolving the dry solids in 4 liters distilled water: 20 g NTA, IDA, or $NaNO_3$, 8 g $NaNO_2$ or NIDA. We gave 100 ml of each solution to 5 rats in a cage 5 nights a week. The solutions were usually consumed by morning, and the animals were given tap water during the day and on weekends. Treatment with NIDA was for 72 weeks; all other solutions were given for 84 weeks. Animals, dead or killed at the termination of the experiment (104 weeks after the beginning of treatment), were completely autopsied. All tumors observed grossly were confirmed by microscopic examination.

RESULTS

There was no sign of toxic effect from any treatment, and most animals survived until the end of the experiment. The pathologic findings are given in table 1, in which the tumors are listed by organ. All tumors were of the type occasionally found in untreated controls of this rat strain.

DISCUSSION

No significant difference in tumor incidence or organ distribution of tumors was apparent between the rats fed NTA or its derivatives and the controls. Surprisingly, NIDA was noncarcinogenic, even though each animal received more than 14 g of the compound (30–60 g/kg body weight) during 72 weeks. In this, NIDA resembles another nitrosamino acid, nitrosoproline (*3*) and differs from the carcinogenic nitrososarcosine (*3*) that could be derived from NIDA by decarboxylation. Because of the lack of activity of NIDA in rats, the formation of NIDA from IDA or NTA and nitrite in the stomach is not biologically significant. Similarly, the possible reduction of nitrate to nitrite was unnoticed and is unimportant here, though it might be important when amines that are precursors of carcinogenic nitrosamines are ingested at the same time.

The high incidence of tumors of "normal" type in both experimental and control groups of these rats is unexpected and difficult to explain. Nevertheless, the absence of tumors of the type commonly induced in rats by nitrosamines (*3*) (in liver, kidney, lung, esophagus) shows that NTA is not a carcinogenic hazard, despite the consumption by the animals of more than 40 g NTA or IDA, a considerable proportion of the body weight. Our groups of animals were small, and statistical analysis of the results (by Mr. T. J. Mitchell of the Statistics Department, Mathematics Division, Oak Ridge National Laboratory) could not be conclusive, though no difference in tumor incidence between groups was deduced. Tests on a larger scale might reveal a tumorigenic effect of NTA and associated compounds, but such an effect would be slight.

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TABLE 1.—*Tumors in rats treated with NTA and derivatives in drinking water*

Treatment	Daily dose (mg)	Total dose (g)	Number of rats			Number of rats with tumors of						Other tumors
			Total	TB*	TF*	Pituitary	Breast	Testis	Uterus/vagina	Adrenal	Pancreas	
NTA	100	42	15 ♂	9	6	2	—	4	—	1	2	1 kidney, 1 lymphoma, 1 lymph node.
			15 ♀	7	8	4	—	—	2	1	—	2 lymphoma.
$NTA+NaNO_2$	100	42	15 ♂	8	7	2	—	1	—	3	—	3 parathyroid, 2 kidney.
	40	16	15 ♀	9	6	5	—	—	4	1	—	1 lymphoma, 1 zymbal gland, 1 ovarian tube, 1 ear.
IDA	100	42	15 ♂	8	7	4	1	—	—	4	1	1 salivary gland, 1 lymphoma.
			15 ♀	10	5	6	2	—	3	1	—	
$IDA+NaNO_2$	100	42	15 ♂	7	8	1	—	1	—	—	3	1 kidney, 1 brain, 1 sarcoma, 1 eyelid.
	40	16	15 ♀	10	5	5	1	—	3	4	—	1 forestomach, 1 liver, 1 skin, 1 thymoma, 1 heart.
$IDA+NaNO_3$	100	42	15 ♂	9	6	3	—	3	—	2	—	1 leiomyosarcoma.
	100	42	15 ♀	11	4	5	4	—	3	5	1	1 forestomach, 1 fallopian tube.
NIDA	40	14. 4	15 ♂	8	7	5	—	—	—	3	3	1 muscle.
			15 ♀	9	6	4	—	—	1	3	—	1 skin.
$NaNO_3$	100	42	15 ♂	6	9	—	—	2	—	2	2	1 heart.
			15 ♀	12	3	11	3	—	1	2	1	1 skin, 1 brain.
$NaNO_2$	40	15	15 ♂	9	6	1	—	2	—	1	1	3 skin, 3 soft tissue.
			15 ♀	10	5	6	2	—	1	—	—	1 skin, 1 stomach, 1 thymus.
Untreated (control)			15 ♂	5	10	—	—	3	—	—	1	1 soft tissue.
			15 ♀	4	11	3	—	—	1	—	—	

* TB = tumor-bearing; TF = tumor-free.

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Changes in Plasma and Muscle Creatine Concentration after Increases in Supplementary Dietary Creatine in Dogs[1]

John A Lowe,[2] Martin Murphy and Victoria Nash

Gilbertson & Page Ltd., Welwyn Garden City, Herts, AL7 1LF, UK

EXPANDED ABSTRACT

KEY WORDS: • *dogs* • *creatine* • *muscle* • *plasma* • *creatinine*

Creatine (Cr)[3] is an important component of the energy delivery process in tissues with a high and/or fluctuating energy demand. In the phosphorylated form, phosphocreatine (PCr) is directly involved in maintaining low adenosine diphosphate concentrations at the sites of energy utilization. Consequently, the maintenance of an adequate supply of PCr at the contractile site of the muscle is important for the perpetuation of muscle performance during intense exercise and exertion.

In normally active animals and humans, a sufficient supply of PCr is considered to be synthesized from the amino acids arginine and glycine (Harris and Lowe 1995). It has been observed in humans, however, that supplementary dietary Cr can, over time, increase the normal muscle content of 100–130 mmol/kg dry weight by between 2 and 40 mmol/kg (Harris et al. 1992). Dietary Cr supplementation has been shown to have a positive effect on short-lasting maximal exercise performance and sustained intense exercise with reduced metabolic effort (Balsom et al. 1993, Greenhaff et al. 1993, Harris et al. 1993); therefore, such Cr loading might be considered beneficial to the working and/or sporting dog.

In the wild, the intake of Cr by the dog from freshly killed prey can be regular and substantial, 1.23–3.0 mmol/(kg body weight · d) (Rohrs 1987). However, a recent review of the dietary intake of Cr in the domestic dog indicated that dietary supply may be limited (Harris et al. 1997). Although this should not be regarded as a deficiency of the diet, it is certainly possible that an increase in supply may influence performance as with human athletes. A recent study in humans showed that long-term, low dose supplementation of the diet with Cr was ultimately as effective in raising muscle Cr concentration as a short-term, high dose supplementation (Hultman et al. 1996). In dogs, it has been shown that Cr is equally well absorbed into the plasma from either fresh meat or synthetic Cr (Harris and Lowe 1995). It remains unknown, however, which of the feeding regimens is the most appropriate to maximize muscle Cr concentration in dogs.

A preliminary study was therefore conducted to investigate the effect of increasing dietary supplemental Cr on muscle and plasma Cr concentrations in dogs before a further more detailed study of the accumulation of muscle Cr over time.

Materials and methods. Twelve adult Beagle dogs (13.7 kg, SD 2.59) were housed in 2.4 m^2 (1.7 m × 1.4 m) concrete block pens with an open-mesh steel gate to the front allowing them visual access to kennel-mates and the central walkway/exercise area. Bedding was provided in the form of soft-wood shavings. The kennel building was heated and ventilated to maintain a temperature between 16 and 24°, 30–70 % relative humidity with 12 h of light in a 24-h period. All dogs were monitored throughout the day and pens cleaned once daily. The study protocols were appropriately approved and the animals maintained under the care of a veterinary surgeon for the duration of the study in compliance with the 1986 EC directive (86/609/EEC) regarding the protection of animals used for experimental and other scientific purposes. All dogs had been fed a basal diet (Lowe et al. 1994), which contained 0.156 mmol Cr/kg diet for at least 2 mo. The dogs were then randomly allocated to four treatment groups ($n = 3$ per group) and the diet supplemented with dry crystalline creatine monohydrate to provide the equivalent of 0.38, 0.76, 1.53 or 3.05 mmol Cr/kg body weight daily for 28 d. Muscle biopsy samples were taken under anesthesia from the biceps femoris muscle of each dog using a percutaneous needle biopsy technique on d 0 (the day preceding Cr supplementation), 14 and 28; samples were frozen immediately. Subsequently, the samples were freeze-dried, dissected from visible connective tissue, powdered and analyzed for Cr, creatinine (Cn) and PCr, using reverse-phase ion-pairing high performance liquid chromatography (Dunnett et al. 1991). Blood samples were taken immediately before feeding and then at 0.5, 1, 2, 3, 4 and 5 h after feeding on d 0, 14 and 28. All samples were immediately centrifuged and the plasma separated and frozen for subsequent Cr and Cn analysis.

Plasma Cr and Cn were analyzed by ANOVA using the area under the plasma curve for 5 h after feeding (AUC_5) within each collection day. With the use of ANOVA, muscle

[1] Presented as part of the Waltham International Symposium on Pet Nutrition and Health in the 21st Century, Orlando, FL, May 26–29, 1997. Guest editors for the symposium publication were Ivan Burger, Waltham Centre for Pet Nutrition, Leicestershire, UK and D'Ann Finley, University of California, Davis.

[2] To whom correspondence should be addressed.

[3] Abbreviations used: AUC_5, the areaunder the plasma curve, calculated by integration, for the 5 h after feeding for plasma creatine and/or creatinine content in mmol; Cn, creatinine; Cr, creatine; PCr, phosphocreatine; TCr, the sum of Cn plus Cr plus PCr in muscle expressed in mmol/kg.

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TABLE 1

The mean and SEM for AUC_5 values for plasma Cr and Cn in dogs together with the time of occurrence of peak plasma Cr and Cn concentration in hours after feeding dietary supplemental Cr for 14 or 28 d at 0.38, 0.75, 1.53 or 3.05 mmol/(kg body weight · d)[1]

	Plasma creatine (Cr)				Plasma creatinine (Cn)			
	Mean	SEM	P	Peak	Mean	SEM	P	Peak
Day 14								
0.38 Treatment	227.2	49.76	0.009	1	111.7	22.04	0.41	2
0.75 Treatment	289.6			1	146.5			2
1.53 Treatment	366.0			1	115.3			1
3.05 Treatment	639.6			1	132.1			1
Day 28								
0.38 Treatment	253.2	64.64	0.005	0.5	88.5	39.2	<0.001	1
0.75 Treatment	294.1			0.5	113.1			1
1.53 Treatment	482.1			1	201.6			1
30.5 Treatment	849.0			3	348.2			3

[1] AUC_5, the area under the plasma curve for the 5 h after feeding.

Cr concentration was examined by regression on Cr intake within a sample day. It is acknowledged that simple ANOVA between the two chosen time points is inappropriate; therefore, changes in muscle Cr concentration over time within the experiment were examined by a regression model.

Results. Significant differences were observed in the AUC_5 for plasma Cr on d 14 (P = 0.009) and on d 28 (P = 0.005) (**Table 1**). The 1.53 and 3.05 mmol/kg treatments substantially increased the AUC_5 and peak plasma values for Cr. The profiles for plasma Cr were similar in shape for both d 14 and 28. Curves for the pooled data are shown for illustration in **Figure 1**. The plasma Cn profile for each treatment closely followed the plasma Cr profile with no apparent time lag. Although the plasma Cn profile was similar in shape, there was greater variability in value. There was a correlation between the AUC_5 for plasma Cn and the AUC_5 of Cr, which could be explained by the following equation:

$$\text{Plasma Cn} = [0.32(\text{SEM} = 0.096) \times \text{Plasma Cr}] - 21.1\ (\text{SEM} = 47.32) \quad R = 0.81$$

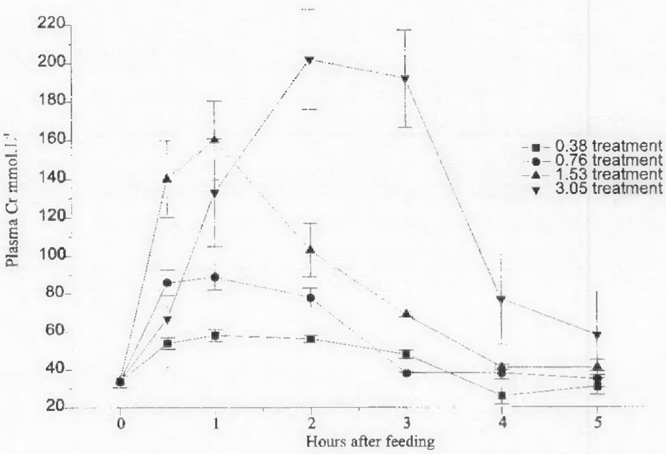

FIGURE 1 The composite plasma creatine profiles from d 14 and 28 expressed as mean (± SEM) plasma creatine (mmol/L) in dogs (3 per treatment) over the 5 h after a meal supplemented with 0.38, 0.76, 1.53 or 3.05 mmol creatine/kg body weight.

The concentrations of the individual components, Cr, PCr and Cn found in the muscle samples were different from that expected to be found in vivo. In all probability, this is due to the likely hydrolysis of PCr during sample collection and preparation. The data were therefore analyzed on the basis of the total PCr + Cr + Cn, termed TCr, in the muscle sample. The TCr found in the muscles of supplemented dogs was within the range found in the muscles of Cr-supplemented humans. Variation in sample adenosine triphosphate (mean 23.9 ± 0.45 mmol/kg) indicated that the samples were consistent in their muscle content and did not account for any differences seen in TCr among dogs. Numerical increases, on the order of 8–32 mmol/kg in muscle TCr were observed within individual dogs from d 0 to 14. Further increases on d 28 were not observed, and no consistent effect over time for any treatment could be established. The mean values for each day and treatment are shown in **Table 2**. Large between-animal variation was observed both pre- and post-treatment, which, together with the small sample size, accounted for the overall large P-values reported for the treatment effects.

Dogs with low initial muscle TCr increased their muscle

TABLE 2

The effect of creatine (Cr) intake on muscle Cr concentration. Means (mmol/kg dry muscle) and SEM of PCr + Cr + Cn (TCr) concentration in the muscle of dogs before (d 0) and 14 and 28 d after dietary supplementation with 0.38, 0.76, 1.53 or 3.05 mmol Cr/kg body weight

	Mean	SEM	P	n
Day 0				
Basal diet	128.6	17.19		12
Day 14				
0.38 Treatment	149.4	19.93	0.23	3
0.76 Treatment	126.5			3
1.53 Treatment	144.1			3
3.05 Treatment	110.8			3
Day 28				
0.38 Treatment	131.8	15.42	0.29	3
0.76 Treatment	117.2			3
1.53 Treatment	134.1			3
3.05 Treatment	106.1			3

TABLE 3

The effect of supplementary Cr on muscle TCr concentration by repeated ANOVA for all data and excluding dogs with high (>140 mmol kg) initial muscle Cr[1]

	Intercept	Slope	SEM	*P*	*R*	*P*	*n*
All dogs	128.6	−3.7	2.55	0.16	0.27	0.16	12
Dogs <140 mmol Cr kg	111.7	11.8	3.44	0.003	0.92	0.006	9

[1] Cr, creatine; TCr, the sum of creatinine plus Cr plus phosphocreatinine.

TCr concentration more than those with higher initial muscle TCr concentrations. Within a given day, there were between-treatment differences, with the 3.05 mmol/kg treatment producing consistently lower muscle Cr values.

Elimination of the dogs ($n = 3$) with high (>140 mmol/kg) initial muscle TCr concentrations from the data resulted in a more dramatic treatment effect by reducing the mean and the variability (SEM) of the TCr in the control dogs from 128.6 ± 17.19 to 111.7 ± 5.2. This resulted in a more definite effect of Cr intake on muscle Cr concentration, (**Table 3**). The observed increase, similar to that in humans (Hultman et al. 1996), is likely to be sufficient to account for the improvement in lactate threshold observed in dogs fed dietary supplemental Cr (Lowe, unpublished observations).

Discussion. The data indicate that there may be large differences in muscle TCr concentration in dogs despite a constant dietary supply. The effect of feeding dietary supplemental Cr over a 4-wk period is to increase the muscle TCr of those dogs with low (<140 mmol/kg) initial TCr while having a negligible effect on those dogs with higher initial TCr values. Within the confines of the number of animals used, the data appear to suggest that any increase in muscle TCr concentration is maximized within 14 d and that this effect is achieved, in this study, by supplementing the diet with as little as 0.38 mmol Cr/kg body weight. This is consistent with recent data reported for humans (Hultman et al. 1996). There appears to be little benefit from feeding larger amounts of supplemental dietary Cr in terms of further increasing muscle TCr concentration after 14 d. However, on the basis of the data reported by Hultman et al. (1996) for humans, the feeding of supplementary Cr in excess of 0.38 mmol/kg may result in consistently higher muscle TCr concentration being attained sooner than 14 d after the start of the feeding regimen.

The plasma Cn content, by d 28, increased in relation to the amount of supplementary dietary Cr fed and as a proportion (0.39 ± 0.015) of plasma Cr. This increase in plasma Cn observed with increases in supplementary dietary Cr will result in increases in urinary Cn excretion in dogs supplemented with dietary Cr. The results of this preliminary study indicate that further studies are required to expand our knowledge of Cr metabolism in the dog.

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PERSPECTIVES

OPINION

Nitrate, bacteria and human health

Jon O. Lundberg, Eddie Weitzberg, Jeff A. Cole and Nigel Benjamin

Nitrate is generally considered a water pollutant and an undesirable fertilizer residue in the food chain. Research in the 1970s indicated that, by reducing nitrate to nitrite, commensal bacteria might be involved in the pathogenesis of gastric cancers and other malignancies, as nitrite can enhance the generation of carcinogenic *N*-nitrosamines. More recent studies indicate that the bacterial metabolism of nitrate to nitrite and the subsequent formation of biologically active nitrogen oxides could be beneficial. Here, we will consider the evidence that nitrate-reducing commensals have a true symbiotic role in mammals and facilitate a previously unrecognized but potentially important aspect of the nitrogen cycle.

In an adult human, prokaryotic cells easily outnumber the eukaryotic cells as ~90% of all cells in the body are bacteria[1]. Most of these bacteria reside in the gastrointestinal tract, with the highest density found in the oral cavity and the large intestine[1]. The complex interplay between the host and the micro-organisms that inhabit the mucosal and epithelial surfaces has been intensely studied. That this relationship can be symbiotic is clearly illustrated, for instance, by vitamin K synthesis, which requires colonic bacteria[2]. A proper balance between the different species competing for space in the gut is also essential, and disturbances in the flora, as a result of excessive use of antibiotics for example, can cause severe infections with opportunistic pathogens such as *Clostridium difficile*[3].

In the gastrointestinal tract a relationship has been observed between *Helicobacter pylori* infection and gastric cancer[4,5]. The commensal flora has also been implicated in the pathogenesis of gastric cancer and other malignancies and, for gastric cancer, much research has focused on the nitrate-reducing bacteria[6–11]. Facultative anaerobic bacteria in the oral cavity reduce salivary nitrate to nitrite[12] and this nitrite enhances the gastric generation of *N*-nitrosamines, a versatile class of carcinogens in animals[13]. However, despite extensive research over the past 50 years, the link between commensal bacteria, nitrate and human gastric cancer is still unclear[14–18]. In fact, there is increasing evidence that the nitrite that is formed in the mouth can subsequently be used by the host to form biologically useful nitrogen oxides (including nitric oxide, NO) that are important for host defence and for the maintenance of normal physiological homeostasis in the stomach and elsewhere[17,19–26]. This newly described pathway for the generation of reactive nitrogen intermediates (RNIs) in mammals complements the production of RNIs by nitric oxide synthase (NOS) in white blood cells[27–29].

Here, we consider the possibility that nitrate-reducing bacteria on mucosal surfaces are truly symbiotic and suggest that the previous view that dietary nitrate has only harmful effects should be reconsidered. We will present evidence that there is an active nitrogen cycle in mammals that depends on bacterial enzymes and which results in the generation of biologically active nitrogen oxides with possible beneficial effects.

Sources of nitrate and its circulation

Exogenous sources of nitrate and nitrite. The main dietary source of nitrate (NO_3^-) is vegetables, which account for 60–80% of the daily nitrate intake in people on a typical western diet[30]. Nitrite (NO_2^-) is also found in some foodstuffs — for example, it is used as a food additive in meat to prevent botulism and to enhance its appearance. Other environmental sources of nitrate and nitrite include cigarette smoke[31] and car exhausts. These and other environmental pollutants contain volatile nitrogen oxides, some of which are converted to nitrate or nitrite in the body.

Endogenous sources of nitrate. The main source of endogenous nitrate in mammals is the L-arginine-NO pathway[32], which is constitutively active in numerous cell types throughout the body. NO is produced from the amino acid L-arginine and molecular oxygen by NO synthases (NOSs). The general biological implications of NO have been reviewed extensively elsewhere[33,34]. Although in simple aqueous systems NO is oxidized to nitrite, in mammals NO predominantly reacts with oxidized haemoglobin and other compounds to form nitrate[35] (BOX 1). Under basal conditions, the metabolites of endogenous NO in plasma are mainly derived from the L-arginine-NO pathway in the endothelium of blood vessels[36–38] and possibly neuronal tissue. However, during systemic inflammatory reactions or infections, white blood cells and other cells express an inducible NOS (iNOS), which produces large amounts of NO and ultimately leads to a considerable increase in the concentrations of nitrate in plasma[39–41].

The entero-salivary circulation of nitrate. The metabolic fate of nitrate in humans is outlined in FIG. 1. After ingestion, nitrate is rapidly and effectively absorbed proximally from the gastrointestinal tract into the bloodstream, where it mixes with endogenously synthesized nitrate. Peak plasma concentrations are seen within 60 minutes of nitrate ingestion and the half-life of nitrate in plasma is about 5 hours[42]. Most nitrate is ultimately excreted in the urine but some is excreted in the saliva, sweat and possibly also the

Box 1 | The biological chemistry of some reactive nitrogen intermediates

Nitric oxide synthesis

$$\text{Arginine} + O_2 \rightarrow \underset{\text{Nitric oxide}}{NO} + \text{Citrulline}$$

Five-electron reduction of the amino acid arginine catalysed by nitric oxide synthase (NOS).

Nitric oxide oxidation

$$\underset{\text{Nitric oxide}}{NO} + \underset{\text{Superoxide}}{O_2^-} \rightarrow \underset{\text{Peroxynitrite}}{ONOO^-}$$

$$\underset{\text{Peroxynitrite}}{ONOO^-} \rightarrow \underset{\text{Nitrate}}{NO_3^-}$$

$$\underset{\text{Peroxynitrite}}{ONOO^-} + H^+ \rightarrow \underset{\text{Peroxynitrous acid}}{HONOO} \rightarrow \underset{\text{Hydroxyl radical}}{OH^\bullet} + \underset{\text{Nitrogen dioxide}}{NO_2}$$

Nitric oxide reacts very rapidly with the superoxide radical to form the reactive intermediate peroxynitrite, which can isomerize to nitrate or can be protonated to form peroxynitrous acid. Peroxynitrous acid in turn can split into hydroxyl and nitrogen dioxide radicals.

$$\underset{\text{Nitric oxide}}{NO} + \underset{\text{Oxyhaemoglobin}}{Hb^{2+}O_2} \rightarrow \underset{\text{Nitrate}}{NO_3^-} + \underset{\text{Methaemoglobin}}{Hb^{3+}}$$

Nitric oxide is also rapidly oxidized by haemoglobin in red blood cells to form methaemoglobin, which in turn is reduced to normal haemoglobin by the enzyme methaemoglobin reductase.

Nitrate reduction

$$\underset{\text{Nitrate}}{NO_3^-} + e^- + 2H^+ \rightarrow \underset{\text{Nitrite}}{NO_2^-} + H_2O$$

Nitrate is reduced by a bacterial nitrate reductase. Facultative anaerobic bacteria use nitrate as an alternative electron acceptor to oxygen under hypoxic conditions.

Nitrite acidification

$$\underset{\text{Nitrite}}{NO_2^-} + H^+ \rightarrow \underset{\text{Nitrous acid}}{HNO_2} \ (pKa = 3.2)$$

$$\underset{\text{Nitrous acid}}{2HNO_2} \rightarrow \underset{\text{Dinitrogen trioxide}}{N_2O_3} + H_2O$$

$$\underset{\text{Dinitrogen trioxide}}{N_2O_3} \rightarrow \underset{\text{Nitric oxide}}{NO} + \underset{\text{Nitrogen dioxide}}{NO_2}$$

Nitrite is protonated under acidic conditions (such as those in the stomach) to generate nitrous acid, which will spontaneously yield dinitrogen trioxide, nitric oxide and nitrogen dioxide. Dinitrogen trioxide is a powerful nitrosating agent.

Nitrosation

$$\underset{\text{Dinitrogen trioxide}}{N_2O_3} \rightarrow \underset{\text{Nitrosonium ion}}{NO^+} + \underset{\text{Nitrite}}{NO_2^-}$$

$$\underset{\text{Nitrosonium ion}}{NO^+} + \underset{\text{Reduced thiol}}{RSH} \rightarrow \underset{\text{Nitrosothiol}}{RSNO} + H^+$$

$$\underset{\text{Nitrosonium ion}}{NO^+} + \underset{\text{Secondary amine}}{RR'NH} \rightarrow \underset{N\text{-nitrosamine}}{RR'NNO} + H^+$$

Dinitrogen trioxide is a powerful NO^+ donor which can transfer to a great variety of organic side-groups, especially thiols to form *S*-nitroso compounds and secondary amines to form potentially toxic *N*-nitrosamines.

intestines. The exact fate of all of the nitrate in the body is still unresolved as only 60% of isotopically labelled administered nitrate is recovered in the urine[43]. For as-yet-unknown reasons, the concentrations of nitrate excreted in saliva are exceptionally high; up to 25% of plasma nitrate is actively taken up by the salivary glands and secreted with saliva[12], and the resulting salivary nitrate concentrations are at least 10 times higher than the concentrations in plasma. If we were all 'germ-free' and lived in a sterile environment, almost all nitrate — ingested or produced endogenously — would eventually leave the body unaltered because human cells cannot metabolize this anion. However, in reality, some of the sites where nitrate is excreted are heavily colonized by bacteria and, in contrast to mammalian cells, many of these microorganisms express enzymes that can effectively reduce nitrate. Later, we will consider the relevance of bacterial nitrate metabolism for human health but before this, the general handling of nitrogen by bacteria in nature will be described.

Bacterial nitrate metabolism

The biological nitrogen cycle. FIGURE 2 shows a classical, but simplified, overview of the biological nitrogen cycle in which bacteria have a dominant role. In aerobic soils and sediments (and also in conventional wastewater treatment plants), ammonia that is released from human and animal excrement or applied as fertilizer is oxidized by aerobic nitrifying bacteria to nitrate. Nitrate is then reduced to dinitrogen gas by anaerobic denitrifying bacteria, in a process that also requires a source of electrons. Denitrification dominates poorly aerated soils and sediments and is exploited in wastewater treatment. Life would be impossible if the combined processes of nitrification and denitrification were not compensated by a third process, dinitrogen fixation, which completes the nitrogen cycle[44].

Nitrate metabolism in bacteria. Despite the importance of nitrification and denitrification to agriculture and the regeneration of potable water, neither process has significant relevance to human health. Almost without exception, commensal bacteria do not denitrify nitrate but instead catalyse a short-circuit in the nitrogen cycle — the rapid, anaerobic reduction of nitrate via nitrite to ammonia (FIG. 2) — in which the abundant supply of electrons that is provided by anaerobic carbon-rich environments is exploited when the normally more-favoured oxygen is not available[45,46]. Superficially, this process seems to be similar to nitrate assimilation, which is the process by which plants, many eukaryotic microorganisms and some bacteria use nitrate as a source of organic nitrogen compounds, but both the enzymes involved and their transcriptional control are distinct for the two processes. As a broad generalization, the respiratory reduction of nitrate to ammonia is repressed by the presence of oxygen but is insensitive to the availability of ammonia or organic nitrogen compounds. It is induced during anaerobic growth and further

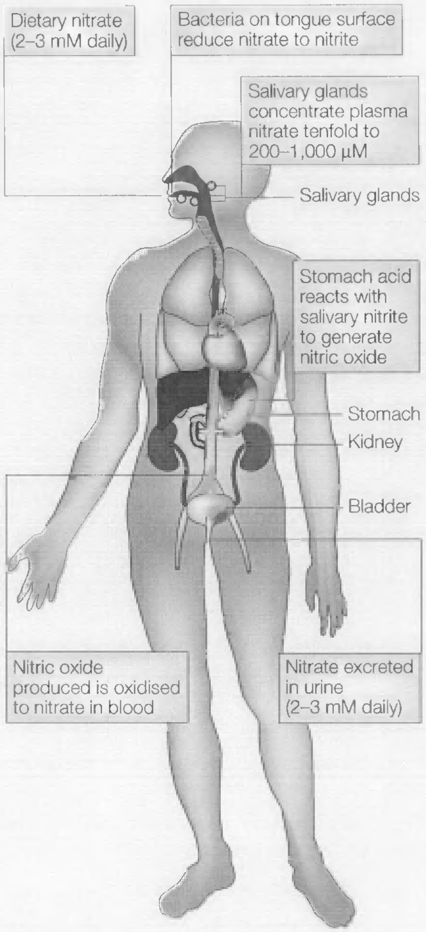

Figure 1 | **The entero-salivary circulation of nitrate in humans.** Vegetables, the main dietary source of nitrate, account for 60–80% of the daily nitrate intake (2–3 mM total) in people on a typical western diet[30]. Once ingested, nitrate is rapidly absorbed from the gastrointestinal tract and mixes with endogenously synthesized nitrate, which mainly comes from the L-arginine-NO pathway[32]. Most nitrate is ultimately excreted in the urine. Up to 25% of plasma nitrate is actively taken up by the salivary glands and secreted with saliva[12]; the resulting salivary nitrate concentrations can be at least 10 times higher than the concentrations in plasma. Salivary nitrate is reduced to nitrite by commensal bacteria in the oral cavity. In the acidic stomach, salivary nitrite is reduced to NO and other RNIs.

induced by the presence of nitrate or nitrite. Conversely, expression of the genes for nitrate assimilation is insensitive to the availability of oxygen, but is tightly regulated by the nitrogen status of the cell[47,48].

Multiple enzymes catalyse parallel pathways. A striking feature of respiratory bacterial nitrate metabolism is that multiple enzymes can catalyse each reaction. At least three types of nitrate reductase catalyse the reduction of nitrate to nitrite[49]. First, a soluble, assimilatory nitrate reductase (NAS) is found in the cytoplasm. Second, energy-conserving nitrate reductases (NAR, for example, NarG, which is encoded by the first gene of the *narGHJI* operon) with catalytic sites located in the cytoplasm are associated with the cytoplasmic membrane, from which they receive electrons for nitrate reduction[50]. Third, soluble, periplasmic nitrate reductases (NAP) are found in many Gram-negative bacteria. All three types are molybdoproteins and some bacteria, for example, *Paracoccus pantotrophus*, possess all three[51]. *Escherichia coli* and *Salmonella enterica* serovar Typhimurium also synthesize three different nitrate reductases: although they lack an assimilatory nitrate reductase, the genes that encode the membrane-associated nitrate reductase are duplicated (the *narG* and *narZ* operons) and differentially regulated[50,52], and they also express a periplasmic nitrate reductase, Nap, which is encoded by the *napFDAGHBC* operon.

Bacterial nitrite reduction. Nitrite reduction is the reaction that defines whether bacteria catalyse denitrification or nitrate reduction to ammonia, and in each case, two distinct classes of nitrite reductase are involved[49]. All nitrite reductases are synthesized preferentially during anaerobic growth. The denitrification of nitrite to nitric oxide is catalysed by the copper-containing NirK or the cytochrome *cd* nitrite reductase NirS, both of which are located in the periplasm[50]. Two biochemically distinct nitrite reductases catalyse the reduction of nitrite to ammonia. The NADH-dependent NirBD nitrite reductase reduces nitrite directly to ammonia in the cytoplasm of some bacteria (for example, Gram-negative enteric bacteria and Gram-positive bacteria such as *Staphylococcus carnosus* and *Bacillus subtilis*). The role of NirBD is to detoxify nitrite that is generated by NarG (the membrane-associated nitrate reductase) during anaerobic growth in the presence of nitrate concentrations that are much greater than those found in warm-blooded animals[52]. More widely distributed is the cytochrome *c* nitrite reductase Nrf, which catalyses the reduction of nitrite to ammonia in the periplasm of Gram-negative bacteria. This enzyme is the terminal component of an electron-transfer pathway in which electrons are transferred from physiological substrates, especially formate (hence the designation Nrf, for nitrite reduction by formate). Nap, the periplasmic nitrate reductase, and Nrf, the periplasmic nitrite reductase, are coordinately regulated to provide a pathway for the reduction of nitrate to ammonia in the periplasm.

It is rare for any single species to be able to catalyse both denitrification and nitrate reduction to ammonia. Although there are also few reports of the occurrence of both NirK and NirS in the same species, some enteric bacteria, such as *E. coli* and *S. typhimurium*, encode both the cytoplasmic nitrite reductase NirBD and the periplasmic nitrite reductase Nrf, and therefore have both a cytoplasmic pathway and a periplasmic pathway for the reduction of nitrate via nitrite to ammonia (FIG. 3). High concentrations of nitrate induce expression of the cytoplasmic pathway, which is encoded by the *narGHJI* and *nirBD* operons, but repress the periplasmic pathway, which is encoded by the *nap* and *nrf* operons. At very low external concentrations of nitrate and nitrite, similar to the concentrations found in body fluids, it is the Nap–Nrf periplasmic pathway that is induced rather than the cytoplasmic pathway. However, it is likely that the cytoplasmic NarG enzyme is responsible for the accumulation of nitrite from nitrate in the mouth.

Chemostat competition experiments have established that the ability to reduce nitrate and nitrite in the periplasm confers a selective advantage relative to a strain that is able to express only the cytoplasmic pathway[52]. Consequently, nitrate reduction in the periplasm of enteric bacteria is believed to be the physiologically significant pathway[53]. How the expression of the genes involved in these

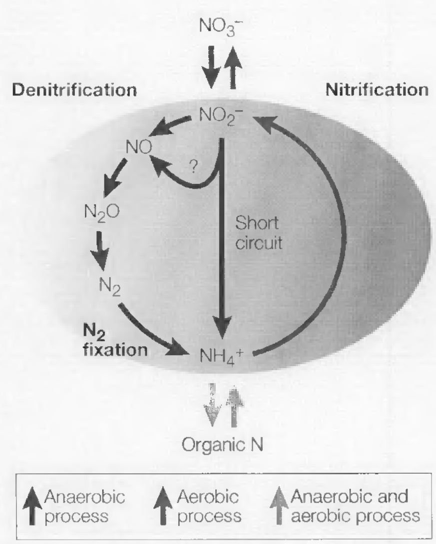

Figure 2 | **The biological nitrogen cycle.** Bacteria have a dominant role in this cycle. Most commensal bacteria do not denitrify nitrate but catalyse a short-circuit in the nitrogen cycle — the rapid, anaerobic reduction of nitrate via nitrite to ammonia. The question mark indicates that the mechanism of NO generation from nitrite during nitrate reduction to ammonia is uncertain.

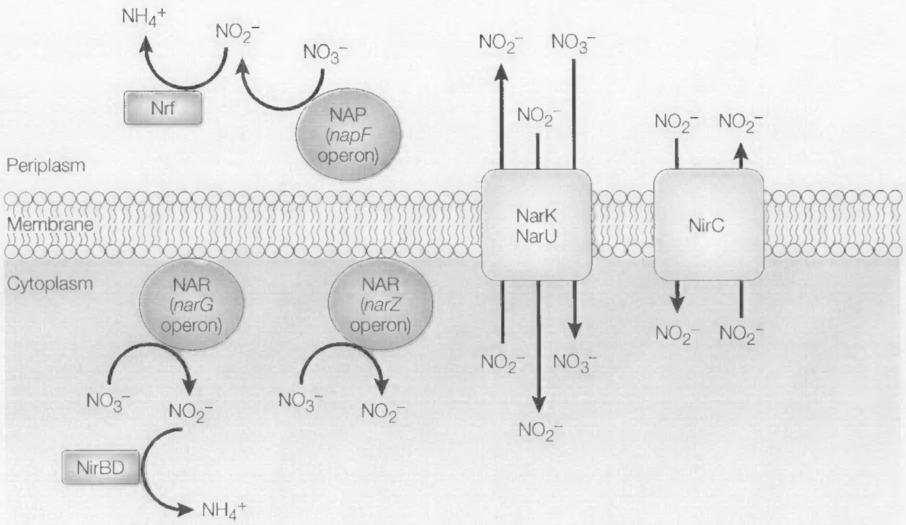

Figure 3 | **Multiple pathways for nitrate and nitrite transport and reduction in *Escherichia coli*.** *E. coli* lacks an assimilatory nitrate reductase, but contains two membrane-associated nitrate reductases, which are encoded by the *narG* and *narZ* operons. A periplasmic nitrate reductase, encoded by the *napF* operon, is also present. In the cytoplasm, the NADH-dependent NirBD nitrite reductase reduces nitrite directly to ammonia. In the periplasm, this reaction is carried out by the cytochrome *c* nitrite reductase, Nrf.

different pathways is regulated has recently been reviewed[49]. Even bacteria that lack denitrification pathways and are known to reduce nitrite directly to ammonia generate relatively high concentrations of NO (REF. 54). The question mark in FIG. 2 indicates the unproven possibility that this NO is produced by one of the enzymes that reduce nitrite to ammonia (NrfA or NirBD). Consistent with the proposal that NO is an enzyme-bound intermediate in this mode of nitrite reduction, it is known that NrfA can reduce NO rapidly to ammonia[55].

Bacteria, nitrate and human disease

Over the past 50 years inorganic nitrate has gained a bad reputation, primarily due to its proposed association with the development of cancer[11] and methaemoglobinaemia, which is a condition that mostly affects infants up to 12 months old and is caused by the oxidation, by nitrite or nitric oxide, of haemoglobin in red blood cells to an abnormal form known as methaemoglobin that cannot bind or transport oxygen[56]. As a consequence, regulatory authorities have expended enormous efforts in trying to minimize environmental exposure to this anion. The proposed deleterious effects of nitrate on human health are not primarily related to the nitrate ion itself. In fact, nitrate has a remarkably low toxicity. Instead, the concern about nitrate arises from its conversion to nitrite, a reaction that is catalysed by the bacterial enzymes described above.

Gastric cancer. The metabolism of nitrate and nitrite can result in the formation of *N*-nitrosamines (BOX 1), which are carcinogenic[57,58]. Individuals can also be exposed to preformed *N*-nitrosamines — for example, from the diet, in certain occupational settings, and through the use of tobacco products. The endogenous formation of *N*-nitroso compounds from nitrite in the stomach occurs in at least two ways (FIG. 4; BOX 1). In an acidic stomach, nitrite forms nitrous acid (pKa 3.2), which is not only a nitrosating agent (NO^+ donor), but also spontaneously decomposes to a variety of nitrogen oxides, including the potent nitrosating agents N_2O_3 and N_2O_4. In a situation in which gastric acidity is reduced — for example, by medication or disease — bacteria can colonize the stomach. Some of these bacteria can catalyse the formation of *N*-nitroso compounds from nitrite at neutral pH values. The exact mechanism for this is unknown, but it is believed to involve a bacterial nitrite reductase[59], with excess production of NO or related products. NO itself is not a nitrosating agent[9,60]. However, in the presence of oxygen, NO is oxidized to NO_2 that exists in equilibrium with N_2O_3 and N_2O_4, which in turn react with secondary amines at neutral pH to form *N*-nitroso compounds[10,61].

Reducing human exposure to preformed *N*-nitrosamines and nitrate is one approach to the prevention of cancer, and in many instances this has been successful, although exposure to *N*-nitrosamines in tobacco products is still very high. However, despite efforts to minimize environmental exposure to nitrate, we still have to live with the nitrate that is produced endogenously from the L-arginine-NO pathway. In healthy people on a western diet, this constitutes as much as 50% of the total daily nitrate load. Although the carcinogenic properties of *N*-nitroso compounds have been well established in cell cultures and animal experiments[13], there is still no clear evidence for a link between nitrate intake and gastric cancer in humans. In fact, many studies show either no relationship or even an inverse relationship between a high intake of nitrate and the occurrence of gastric cancer[14–18]. The mechanism for such a protective effect is unknown, but it could be related to the high concentrations of vitamin C and other antioxidants that are found in nitrate-rich foods such as vegetables. It can also be speculated that gastric nitrogen oxides other than *N*-nitrosamines protect the gastric mucosa (see below) and inhibit the growth of *H. pylori*, which has been implicated as a causative factor for the development of gastric cancer in humans[5].

Bladder cancer. Most dietary and endogenous nitrate is excreted in the urine. As urine is normally sterile, no further reduction to nitrite occurs. However, during urinary-tract infections, considerable amounts of nitrite can be formed by the invading bacteria.

Infection with the parasite *Schistosoma haematobium* is an important risk factor for the development of cancer in the urinary bladder, and *N*-nitrosamines have been proposed to have a role in the pathogenesis of this cancer[62,63]. The proposed mechanism is that chronic infection with nitrate-reducing bacteria, which often occurs with the parasitic disease, increases the concentrations of urinary nitrite, thereby enhancing the generation of *N*-nitrosamines. An increased local rate of endogenous NO production during the chronic parasitic infection has also been suggested to promote nitrosamine formation in the bladder[64].

Generating RNIs from nitrite

N-nitrosamines are not the only compounds that are formed in the body from bacterial-derived nitrite. In 1994, it was shown that large amounts of NO are generated *in vivo* from salivary nitrite in the acidic stomach[19,24]. When nitrite is acidified, several other RNIs are also generated, many of which have biological effects[26]. RNIs are also produced by iNOS, for example, in activated white blood cells, where they have an important role in primary host defence[28,33]. So, there are two parallel pathways for the generation of RNIs in mammals.

The chemistry of RNIs in biological systems is very complex and not yet fully characterized[27,65,66]. RNIs include different oxidation states of nitrogenous compounds including $NO^{\bullet}$, $NO_2^{\bullet}$, N_2O_3, N_2O_4, NO_2^-, *S*-nitrosothiols, $ONOO^-$ and NO_3^-. The chemical nature and reactivity of any RNI in a biological system is determined by ambient factors, including oxygen tension, pH, proximity to haem-containing proteins, redox state and thiol concentration. RNIs also interact with reactive oxygen intermediates (ROIs), which are produced in large quantities by activated mammalian phagocytes. The classical example of this is the generation of peroxynitrite ($ONOO^-$) from the reaction of NO with superoxide (O_2^-)[67]. Some of the central reactions involving RNIs in biological systems are shown in BOX 1.

Antimicrobial effects of RNIs. At neutral pH values, nitrite has a limited effect on bacterial growth and can even function as an alternative oxidant under hypoxic conditions (see above). However, with increasing acidity this anion shows potent antimicrobial activity against a wide variety of bacteria and fungi. At a low pH value, nitrite is protonated to nitrous acid (HNO_2), which further decomposes to a variety of RNIs, many of which have antibacterial activity[26,68] (BOX 1).

The exact mechanism for the antibacterial effects of these nitrogen oxides is still not known, but multiple cellular targets are almost certainly involved[27,68,69] (TABLE 1). DNA is one important target, and DNA damage can occur through oxidative damage, deamination, interaction with DNA-repair systems and several other alterations. Both cell-surface and intracellular proteins are also targets of RNI-induced bacterial damage. Interactions with proteins involve reactive thiols, iron–sulphur clusters, haem groups, zinc-containing moieties, tyrosine residues, tyrosyl radicals and amines.

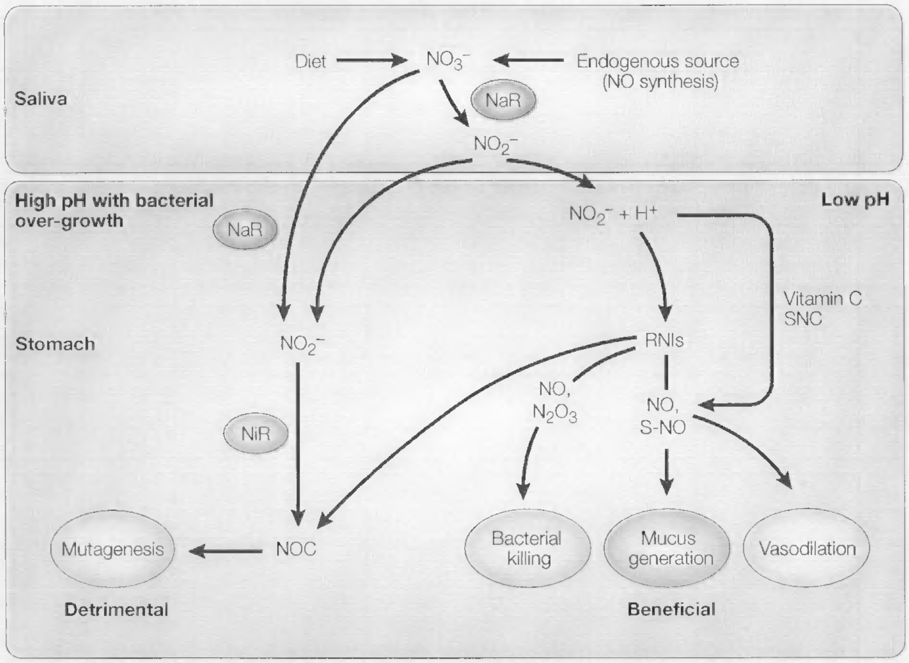

Figure 4 | **The putative effects of nitrite in the stomach.** Bacterial nitrate reductases (NaR) are shown in blue and nitrite reductases (NiR) in red. NOC, *N*-nitroso compounds; RNIs, reactive nitrogen intermediates; SNC, thiocyanate; S-NO, *S*-nitrosothiols.

The sensitivity to any particular nitrogen oxide differs profoundly between different microbial pathogens. For example, $NO^{\bullet}$ is microbicidal for *Mycobacterium tuberculosis*[70], but has low antimicrobial activity against *E. coli*[71–73]. On the other hand, *E. coli* is sensitive to other nitrogen oxides, including *S*-nitrosothiols[74] and peroxynitrite[71], whereas *M. tuberculosis* is not[75].

Susceptibility to the complex mixture of RNIs that is generated from acidified nitrite varies between different species. *H. pylori*[76] and lactobacilli[77] seem to be the least sensitive of the bacteria that have been tested. In addition, *Salmonella*, *Yersinia* and *Shigella* species are more susceptible than *E. coli*[22]. Local environmental conditions, such as the oxygen concentrations, the growth medium and the presence of reducing agents (for example, ascorbic acid or thiocyanate), will also affect the antimicrobial effects of RNIs on a particular microorganism[26,68].

Table 1 | **Proposed mechanisms and cellular targets for antimicrobial effects of reactive nitrogen intermediates (RNIs)**

Targets	Mechanism	Proposed RNI
DNA function		
Oxidative DNA damage (for example, strand breaks, crosslinking or deamination)	*N*-nitrosylation	N_2O_3, $ONOO^-$, $NO_2^{\bullet}$
Filamentation	*S*-nitrosylation	S-NO
Protein function		
Thiol groups (for example, in glyceraldehyde-3-phosphate dehydrogenase, γ-glutamylcysteinyl synthetase)	*S*-nitrosylation, disulphide formation	S-NO, N_2O_3, $NO^{\bullet}$
Haem groups (for example, in cytochromes or catalase)	Nitrosyl-haem formation	$NO^{\bullet}$, S-NO
Iron/zinc-sulphur clusters (for example, in aconitase or DNA-binding proteins)	*S*-nitrosylation	$NO^{\bullet}$, $ONOO^-$
Tyrosine residues (disruption of tyrosine phosphorylation, modification of protein function)	Nitration	$ONOO^-$, $NO_2^- + H_2O_2$
Tyrosyl radicals (for example, in ribonucleotide reductase)	$NO^{\bullet}$ radical interaction	$NO^{\bullet}$
Amines	*N*-nitrosylation	N_2O_3, N_2O_4
Cell wall integrity		
Lipids	Lipid peroxidation	$NO_2^{\bullet}$, $ONOO^-$
Surface thiols	*S*-nitrosylation, disulphide formation	S-NO, N_2O_3, $NO^{\bullet}$

Table 2 | **Examples of how pathogenic and commensal bacteria metabolize nitrite and its reactive derivatives**

Reactive nitrogen species	Protein	Function	References
Escherichia coli			
Nitrite	NrfA NirBD HCP	Nitrite reduction to ammonia Nitrite reduction to ammonia Unknown, but possible HNO_2 reductase	109 110 111
Nitric oxide	NrfA HMP HCP NorV–NorW; flavorubredoxin	Reduces NO to ammonia NO dioxygenase activity Possible NO reductase (?) Alternative NO reductase	55 112,113 Deduced from 111 114
Peroxynitrite	Peroxynitrite reductase; AhpC	Reduces peroxynitrite to nitrite	79
Hydroxylamine	HCP	Reduces NH_2OH to ammonia	80
Campylobacter jejuni			
Nitrite	NrfA	Reduces nitrite to ammonia (?)	Uncharacterized; identified from the genome database
Nitric oxide	NrfA Cgb	Reduces NO to ammonia, as in *E. coli* (?) Possibly detoxifies NO, mechanism unknown	Uncharacterized; identified from the genome database Uncharacterized; identified from the genome database
Neisseria gonorrhoeae			
Nitrite	AniA	Reduces nitrite to NO	115
Nitric oxide	NorB Cytochrome *c'* HMP homologue	Converts NO to N_2O Binds NO at the outer membrane Reduces NO to N_2O (?)	116 117 From incomplete genome database
Hydroxylamine	Cytochrome *c'*		118

HMP, bacterial haemoglobin-like protein; HCP, hybrid cluster protein, also known as the prismane protein.

Bacterial protection against RNIs. Bacteria in the human body — like bacteria in any other natural environment — are exposed to chemical attack by reactive nitrogen (and oxygen) species. It is hardly surprising, therefore, that they have evolved a wide variety of mechanisms to respond to environmental stress. Protection against ROIs has been studied extensively and hundreds of gene products are involved[27]. Despite the obvious implications for human health, it is only recently that the biochemical mechanisms of resistance to RNIs have been studied, and the current picture is incomplete. That NO is toxic to bacteria — and also an obligate intermediate in denitrification — was conclusively established by demonstrating that a mutation in the NO reductase gene was lethal to *Pseudomonas stutzeri* during anaerobic growth in the presence of nitrate or nitrite[78].

Some of the defence systems seem to overlap between ROIs and RNIs, but there are also specific pathways for the detoxification of RNIs. These include indirect pathways such as inhibition of uptake or production of RNIs, repair of RNI-dependent damage and scavenging and detoxification of RNIs and related products[27,68]. The best characterized are the bacterial nitrite and NO reductases, but the importance of NO to human physiology has attracted studies of other mechanisms of NO management. Some examples are listed in TABLE 2. There are a few reports of the identification of bacterial peroxynitrite and hydroxylamine reductases[79,80], but much less is known about the mechanisms by which bacteria detoxify other RNIs. It is also apparent that multiple physiological roles have been assigned to individual proteins. Although the roles of the various proteins from *E. coli* are, with notable exceptions, moderately well defined, few experimental data are available for the corresponding proteins from most other bacteria.

Beneficial effects of acidified nitrite

Killing ingested pathogens. Each day we produce approximately one litre of saliva, which continually flows into the acidic stomach. The levels of NO and other RNIs in the stomach will depend on the nitrite concentration in the saliva. The baseline concentration of NO in the stomach headspace gas is approximately 20 parts per million (ppm)[42]. When consuming a nitrate-free diet, the concentration of nitrite in saliva, even when acidified, is probably not sufficient to kill known enteric pathogens such as *E. coli, Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Enteritidis[19,22]. Following a meal containing high nitrate concentrations (for example, a portion of lettuce), the levels of nitrate and nitrite in saliva increase markedly, resulting in an increase in stomach NO concentrations to >400 ppm[42]. Most enteric pathogens are killed within 1 hour under these conditions *in vitro* (FIG. 5), even if the acid is buffered to pH 3 (REF. 81). This indicates an important role for dietary nitrate in protection against ingested pathogens.

Gastric mucosal integrity. Experimental animal studies[82] as well as clinical studies in humans[83,84] clearly indicate that NO has an important protective role in the stomach, probably by improving gastric mucosal blood flow. The concentrations of NO in the stomach lumen (20–400 ppm) are several orders of magnitude higher than those that are required for vasodilation[85]. As NO is known to easily travel across biological membranes and as NO-donating drugs are gastroprotective, it has been proposed that nitrite-derived NO, acting from the luminal side, could be involved in the regulation of gastric mucosal blood flow (FIG. 4). Several recent studies from different laboratories support this idea. Bjorne and colleagues studied gastric mucosal blood flow and mucus secretion in a rat *in vivo* model after local application of human saliva to the gastric mucosa[20]. Mucosal blood flow and mucus secretion were increased after luminal application of nitrite-rich saliva, whereas saliva from a fasting individual had no effect. These effects were associated with the generation of NO and *S*-nitrosothiols. In addition, pretreatment with an inhibitor of guanylyl cyclase markedly inhibited nitrite-mediated effects on blood flow. This indicates that the observed effects were mediated by NO.

Several other recent animal studies indicate that dietary nitrate has gastroprotective activity through the generation of NO in the stomach[86–88]. Miyoshi *et al.* examined the effects of oral nitrate supplementation on stress-induced gastric injury in rats[86]. Pretreatment with inorganic nitrate was strongly protective and the effects were paralleled by intragastric generation of NO. Interestingly, NO generation and the protective effects of dietary nitrate were abolished when the oral microflora was removed by topical antibiotic treatment before the experiment.

Taken together, these studies clearly indicate that dietary nitrate has important gastroprotective effects. The crucial step in the bioactivation of inorganic nitrate is the reduction to nitrite, which is carried out by the oral microflora.

Oral cavity. As well as acidification of salivary nitrite once it has been swallowed into the stomach, acidification can also occur in the oral cavity itself, which results in the local generation of NO and other RNIs[21,89]. Cariogenic bacteria such as *Streptococcus mutans* and lactobacilli cause dental damage by converting sugars into organic acids. It has now been shown that these microorganisms are susceptible to nitrite and will 'self-destruct' if allowed to produce acid in a nitrite-rich environment[90]. This might explain some of the protective effects of normal saliva against dental caries.

Skin. Normal human skin continually releases NO. Although this could result from NOS that is present in dermal vascular endothelial cells, Weller *et al.* showed that complete inhibition of this enzyme with L-NMMA (a NOS inhibitor) had no effect on the production of NO by the skin[91]. This evidence, together with the observation that antibiotic therapy decreases skin NO formation and applied nitrite enhances NO formation, led to the proposal[91] that commensal bacteria are producing NO by the reduction of sweat nitrate to nitrite and its subsequent conversion to NO by the acidic environment (pH 5.5) on healthy skin. Skin commensal bacteria such as coagulase-negative staphylococci commonly express a nitrate reductase enzyme (M. Wilks, personal communication). The amount of NO and other nitrogen oxides is clearly not sufficient to kill normal skin commensals, but might be useful in suppressing the growth of fungal pathogens, which are more likely to cause disease in this organ[92].

Urine. Most plasma nitrate is eventually excreted in the urine, as discussed above. As urine is sterile, the concentrations of nitrite are usually very low; however, during a lower-urinary-tract infection bacteria can produce nitrite from the surplus nitrate source. In fact, a urinary test-strip for nitrite is routinely used in the clinic to detect urinary-tract infections. Because the pH of infected urine is usually quite high (values of about 6–8), there is normally no further reduction to NO and other RNIs. However, if the pH value of nitrite-containing urine is decreased, large amounts of RNIs are formed. As described above, these nitrogen oxides are bactericidal for several microorganisms. The antibacterial effects of nitrite in urine have been studied *in vitro*[25,93]. All urinary pathogens that have been tested are sensitive to acidified nitrite, including *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus saprophyticus*, and the addition of ascorbic acid to urine further enhances the antibacterial effects[93]. The effects of nitrite are seen when the pH value of urine is decreased to 5.5 or below, which can be achieved by the ingestion of vitamin C or other acidifying agents[93]. Interestingly, acidification of urine is used in traditional folk medicine for the treatment and prevention of urinary-tract infections, although the mechanism of action is poorly understood[93]. It has been proposed that the generation of bactericidal nitrogen oxides in acidified urine contributes to these beneficial effects[93,94].

Nitrite reduction to NO in the systemic circulation. The generation of NO from nitrite occurs spontaneously in highly acidic or reducing environments, as we have discussed. Interestingly, such non-enzymatic generation of NO can also occur systemically. In ischaemic tissues in which the pH value is decreased, NO is formed from nitrite by similar mechanisms[26,95,96]. In addition, recent research indicates that nitrite can be converted to NO by several other pathways, which involve mammalian enzymes or proteins[54,97–104] (TABLE 3). It has now been shown that physiological concentrations of nitrite can dilate blood vessels through conversion to NO[96,99]. With this new knowledge, nitrite might be considered an important vascular storage pool of NO. Interestingly, it was recently found that the levels of nitrite in plasma increase 4–5-fold after ingestion of inorganic nitrate[105]. This increase was abolished if the test subject avoided swallowing after the nitrate intake, thereby illustrating its salivary origin. By extrapolation, this could in fact indicate that the commensal oral flora contributes not only to the local regulation of gastric function, as discussed above, but also to systemic NO-mediated effects, such as the regulation of vascular tone, platelet function[106] and leukocyte adhesion.

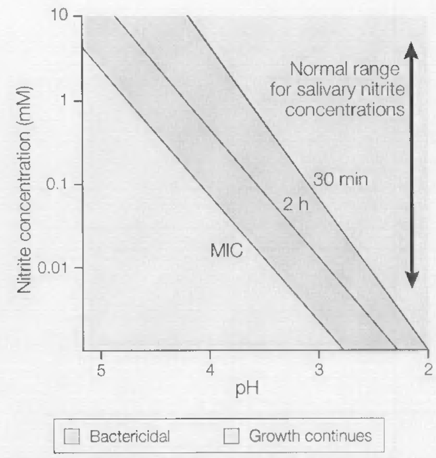

Figure 5 | **The antibacterial effects of salivary nitrite in gastric juice.** The graph shows the minimum bactericidal concentration for 30 min and 2 h and the minimum inhibitory concentration (MIC), all in μmol ml^{-1}, for *Salmonella enterica* serovar Enteritidis exposed to varying concentrations of acid and nitrite. At typical stomach pH values, increasing the nitrite concentration from a low normal salivary concentration to a high normal value renders the acidified nitrite mix bactericidal rather than bacteriostatic. Data taken from REF. 22.

Final speculation and future directions

Nitrate in plasma and tissues represents a stable, inert end product of endogenous and exogenous nitrogen oxides and cannot be further metabolized by mammalian cells. However, as we have described, commensal bacteria can help to 'reactivate' this nitrate by reducing it to the more reactive nitrite. Nitrite can then form various biologically active compounds, including not only potentially harmful *N*-nitrosamines, but also other nitrogen oxides that have possible protective effects.

The newly discovered beneficial effects of nitrite could potentially have therapeutic implications — for example, to selectively increase the blood flow in ischaemic tissues or to stimulate mucosal blood flow locally, for example, in the stomach. Indeed, the generation of NO from nitrite is automatically enhanced in acidic or reducing environments. Another possible development is to use acidified nitrite as an antimicrobial agent — RNIs generated locally, for example, in the stomach, oral cavity, urine[94] or on the skin[107], could be used to treat or prevent infections. Naturally, any beneficial effects of using nitrite or related compounds therapeutically must be verified in controlled clinical studies.

Finally, as described here, it is now clear that there are two pathways for the generation of NO and other RNIs in mammals: the NOS-dependent pathway in phagocytes and other cells and the newly described

Table 3 | **Proposed pathways for the generation of NO from nitrite in mammals**

Location	Source of nitrite	Proposed effects	References
Acidic reduction*			
Stomach	Bacterial reduction of salivary nitrate	Beneficial — host defence, mucosal protection Harmful —carcinogenesis	19–21,24,119
Oral cavity	Bacterial reduction of salivary nitrate	Host defence against peridontal and cariogenic bacteria	90,120
Skin	Bacterial reduction of sweat nitrate	Host defence against skin pathogens	107,121
Lower urinary tract	Bacterial reduction of urinary nitrate	Host defence	93,94,122
Ischaemic tissue	Oxidized NO	Hypoxic vasodilation, ischaemia-reperfusion injury	95,96
Deoxyhaemoglobin			
Blood	Oxidized NO	Vasodilation	97–99
Xanthine oxidoreductase			
Ischaemic tissue	Oxidized NO	Vasodilation	100–102
Milk	Reduction of nitrate in milk	Host defence	103
Cytochrome P450			
Hepatocytes, smooth muscle cells	Oxidized NO, organic nitrates	Final step in NO generation from organic nitrates	98,104
Mitochondrial cytochromes			
Hepatocytes, other cells	Oxidized NO, organic nitrates	Final step in NO generation from organic nitrates	98,104
Bacterial nitrite reductases			
Gastrointestinal tract	Nitrate reductase	Anti-inflammatory activity?	54,123

*With the exception of NO, a variety of different reactive nitrogen intermediates are generated from acidified nitrite (see text and BOX 1 for details).

NOS-independent pathway involving the reduction of nitrite. Although not yet studied specifically, it is possible that these pathways interact *in vivo*. One such interaction can occur in phagocytic cells. The phagosomes of macrophages are very acidic (pH <5), a situation in which nitrite is rapidly converted to toxic RNIs[26,108]. Interestingly, cellular oxygen concentrations affect the two systems differently. Although hypoxia and acidosis inhibit NO generation from iNOS (molecular oxygen is a co-substrate), these conditions greatly enhance the production of RNIs from nitrite. So, the reduction of nitrite by acidification or by other pathways could be a back-up system for the NOS-driven generation of RNIs when conditions for NO production by iNOS are unfavourable. The same could be true for the low-grade generation of NO by constitutive enzymes in blood vessels. When the oxygen supply is insufficient for the NOS to operate (for example, during ischaemia), the nitrite-derived NO pathway automatically takes over.

In mammals, the generation of NO by NOS and its biological significance has been studied intensely over the past two decades. The NOS-independent generation of NO from nitrite is much less well characterized and we have probably only witnessed the start of this fascinating new field. As we have described, bacteria have a pivotal role in this pathway. With the recent identification of potentially important physiological effects of nitrite, the current view of the nitrate-reducing commensals as being only harmful might have to be reconsidered.

Jon O. Lundberg is at the Department of Physiology & Pharmacology, Karolinska Institute, 171 77 Stockholm, Sweden.

Eddie Weitzberg is at the Department of Anaesthesiology and Intensive Care, Karolinska Hospital, 171 76 Stockholm, Sweden.

Jeff A. Cole is at the School of Biochemistry, University of Birmingham, PO Box 363, Edgbaston, Birmingham B15 2TT, UK.

Nigel Benjamin is at the Peninsula Medical School, St. Luke's Campus, Heavitree Road, Exeter EX1 2LU, UK.

Correspondence to J.O.L.
e-mail: jon.lundberg@fyfa.ki.se

doi:10.1038/nrmicro929

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Acknowledgements
The authors wish to thank the Ekhaga Foundation, the Swedish Research Council, the EU 6th Framework Programme and the Swedish Heart and Lung Foundation for generous support.

Competing interests statement
The authors declare competing financial interests: see Web version for details.

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ERRATUM

NITRATE, BACTERIA AND HUMAN HEALTH

Jon O. Lundberg, Eddie Weitzberg, Jeff A. Cole and Nigel Benjamin

Nature Rev. Microbiol. **2**, 593–602 (2004)

Reference 105 was erroneously deleted from the reference list of this article and should have appeared as follows:

105. Lundberg, J. O. & Govoni, M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic. Biol. Med.* **37**, 395–400 (2004).

The enhanced text version of this article has been corrected.

The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics

Jon O. Lundberg, Eddie Weitzberg‡ and Mark T. Gladwin§||*

Abstract | The inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) were previously thought to be inert end products of endogenous nitric oxide (NO) metabolism. However, recent studies show that these supposedly inert anions can be recycled *in vivo* to form NO, representing an important alternative source of NO to the classical L-arginine–NO-synthase pathway, in particular in hypoxic states. This Review discusses the emerging important biological functions of the nitrate–nitrite–NO pathway, and highlights studies that implicate the therapeutic potential of nitrate and nitrite in conditions such as myocardial infarction, stroke, systemic and pulmonary hypertension, and gastric ulceration.

Xanthine oxidoreductase
An enzyme involved in purine metabolism that catalyses the oxidation of hypoxanthine to xanthine and the further oxidation of xanthine to uric acid.

**Department of Physiology and Pharmacology, Karolinska Institute, SE-171 77 Stockholm, Sweden.
‡Section of Anesthesiology and Intensive Care, Department of Physiology and Pharmacology, Karolinska Institute, SE-171 77 Stockholm.
§Pulmonary and Vascular Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Heath, Bethesda, Maryland 20892, USA.
||Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda.
e-mails: jon.lundberg@ki.se; eddie.weitzberg@ki.se; mgladwin@mail.nih.gov*
doi:10.1038/nrd2466
Published online
2 January 2008

Nitrite (NO_2^-) and nitrate (NO_3^-) are known predominantly as undesired residues in the food chain with potentially carcinogenic effects[1,2], or as inert oxidative end products of endogenous nitric oxide (NO) metabolism. However, from research performed over the past decade, it is now apparent that nitrate and nitrite are physiologically recycled in blood and tissues to form NO and other bioactive nitrogen oxides[3–6]. Therefore, they should now be viewed as storage pools for NO-like bioactivity, thereby complementing the NO synthase (NOS)-dependent pathway. The recognition of this mammalian nitrogen cycle has led researchers to explore the role of nitrate and nitrite in physiological processes that are known to be regulated by NO.

The bioactivation of nitrate from dietary or endogenous sources requires its initial reduction to nitrite, and because mammals lack specific and effective nitrate reductase enzymes, this conversion is mainly carried out by commensal bacteria in the gastrointestinal tract and on body surfaces[7,8]. Nitrite is unique to the nitrogen oxides in its redox position between oxidative (NO_2 radical) and reductive (NO radical) signalling and its relative stability in blood and tissue[9]. Once nitrite is formed, there are numerous pathways in the body for its further reduction to NO, involving haemoglobin[6,10], myoglobin[11,12], xanthine oxidoreductase[13–15], ascorbate[16], polyphenols[17,18] and protons[3,4] (BOX 1). The generation of NO by these pathways is greatly enhanced during hypoxia and acidosis, thereby ensuring NO production in situations for which the oxygen-dependent NOS enzyme activities are compromised[19,20]. Nitrite reduction to NO and NO-modified proteins during physiological and pathological hypoxia appear to contribute to physiological hypoxic signalling, vasodilation, modulation of cellular respiration and the cellular response to ischaemic stress[6,11,21–26].

Here, we review the metabolism and biological roles of NO within the body, and discuss the potential therapeutic use of nitrate or nitrite to treat various disorders, including those associated with vasoconstriction or ischaemia–reperfusion, as well as gastric ulcers.

NOS-independent NO generation

The NOS enzymes utilize L-arginine and molecular oxygen to produce the free-radical gas •NO, a critical regulator of vascular homeostasis, neurotransmission and host defence[27,28]. NO is an autocrine and paracrine signalling molecule whose lifetime and diffusion gradients are limited by scavenging reactions involving haemoglobin, myoglobin and other radicals. However, as discussed here, NO can be stabilized in the blood and tissue by oxidation to nitrate and nitrite, which can be considered as endocrine molecules that are transported in the blood, accumulate in tissue and have the potential to be converted back to NO under physiological and pathological conditions.

Interestingly, the L-arginine–NOS pathway is oxygen dependent, whereas the nitrate–nitrite–NO pathway is gradually activated as oxygen tensions falls. In this sense, NOS-independent NO formation (FIG. 1) can be viewed as a back-up system to ensure that there is sufficient NO formation when oxygen supply is limited, which is analogous to the complementary role of anaerobic glycolysis in energetics. The exact oxygen tension at which NOS-dependent NO generation fails to signal is unknown, in part owing to uncertainties about the *in vivo* K_m of

Box 1 | The biological chemistry of some reactions involving nitrite

Nitrite (NO_2^-) is formed in the body via the oxidation of nitric oxide (NO) or through the reduction of nitrate (NO_3^-).The non-enzymatic reaction of NO with oxygen in tissues is relatively slow, whereas its oxidation by the multicopper oxidase ceruloplasmin in plasma is rapid. Commensal bacteria in the oral cavity and gastrointestinal tract contribute to nitrite formation via a one-electron reduction of nitrate.

Reduction of nitrite to NO occurs in blood and tissues and proceeds through several enzymatic and non-enzymatic pathways, some of which are listed below. The acidic reduction of nitrite results in the generation of NO but also other nitrogen oxides, with nitrosating (N_2O_3) and nitrating (nitrogen dioxide, $^{\bullet}NO_2$) properties. In the presence of ascorbic acid or polyphenols, the acidic reduction of nitrite is greatly enhanced with less generation of N_2O_3 and $^{\bullet}NO_2$. Oxidation of nitrite occurs in the red blood cell and results in the formation of nitrate and methaemoglobin (Met-Hb).

Nitrite formation

Auto-oxidation of NO

$4\,^{\bullet}NO + O_2 + 2\,H_2O \rightarrow 4NO_2^- + 4\,H^+$

Ceruloplasmin

$^{\bullet}NO + Cu^{2+} \rightarrow NO^+ + Cu^+$

$NO^+ + H_2O \rightarrow HNO_2 + H^+$

$HNO_2 \leftrightarrow H^+ + NO_2^-$

Bacterial nitrate reductase

$NO_3^- + e^- + H^+ \rightarrow NO_2^- + H_2O$

Nitrite reduction

Deoxyhaemoglobin/myoglobin

$NO_2^- + Fe^{2+} + H^+ \rightarrow {}^{\bullet}NO + Fe^{3+} + OH^-$

Xanthine oxidoreductase

$NO_2^- + Mo^{4+} + H^+ \rightarrow {}^{\bullet}NO + Mo^{5+} + OH^-$

Protons

$NO_2^- + H^+ \rightarrow HNO_2$

$2\,HNO_2 \rightarrow 2\,N_2O_3 + H_2O$

$N_2O_3 \rightarrow {}^{\bullet}NO + {}^{\bullet}NO_2$

Ascorbate

$NO_2^- + H^+ \rightarrow HNO_2$

$2\,HNO_2 + Asc \rightarrow 2\,^{\bullet}NO + dehydroAsc + 2\,H_2O$

Polyphenols (Ph-OH)

$NO_2^- + H^+ \rightarrow HNO_2$

$Ph\text{-}OH + HNO_2 \rightarrow Ph\text{-}^{\bullet}O + {}^{\bullet}NO + H_2O$

Nitrite oxidation

Haemoglobin

$4\,NO_2^- + 4\,HbO_2 + 4H^+ \rightarrow 4NO_3^- + 4\,Met\text{-}Hb + 2\,H_2O + O_2$

Autocrine
A form of hormonal signalling in which a cell secretes a chemical messenger that binds to receptors on the same cell, leading to changes in the cell.

Paracrine
A form of cell signalling in which the target cell is close to (para = alongside of or next to) the signal-releasing cell.

Endocrine
A form of cell signalling in which chemical mediators are released directly into local blood vessels and travel to distant organs to regulate the target organ's function.

the NOS enzymes for oxygen and the fact that the rate of NO oxidative metabolism is reduced at low oxygen. However, it is clear that at very low oxygen tensions NO generation in tissues is independent of NOS activity and dependent on nitrite[5,22,26]. This principle has driven hypotheses that nitrite participates in hypoxic vasodilation and in the regulation of oxygen consumption at the mitochondrial level. It also predicts a role for nitrite in cytoprotective signalling in the setting of pathological ischaemia and reperfusion.

Sources of nitrate and nitrite

There are two major sources of nitrate and nitrite in the body: the endogenous L-arginine–NO synthase pathway and the diet. NO, generated by NOS enzymes, is oxidized in the blood and tissues to form nitrate and nitrite[27]. The reaction of NO with oxyhaemoglobin produces nitrate and methaemoglobin[27], whereas the oxidation of NO forms nitrite, a process that is catalysed in plasma by the multi-copper oxidase and NO oxidase ceruloplasmin[29]. In NOS knockout mice, the circulating nitrite levels are reduced by up to 70%[30], and nitrite levels are also lower in mice and humans lacking ceruloplasmin[29]. Normal plasma levels of nitrate are in the 20–40 μM range, while nitrite levels are substantially lower (50–300 nM)[8,25,31,32]. Regular exercise increases endothelial NOS (eNOS) expression and activity[33], which results in higher circulating levels of nitrate[33–35]. In systemic inflammatory disorders such as sepsis and severe gastroenteritis, nitrate and nitrite levels are greatly increased owing to massive inducible NOS (iNOS) induction[27,36]. By contrast, in diseases with endothelial dysfunction and reduced eNOS activity, plasma levels of nitrate and nitrite are often low[37].

Dietary nitrate intake is considerable and many vegetables are particularly rich in this anion[38]. For example, a plate of green leafy vegetables such as lettuce or spinach contains more nitrate[38] than is formed endogenously over a day by all three NOS isoforms combined[39]. Drinking water can also contain considerable amounts of nitrate, although in many countries the levels are strictly regulated. Nitrite can be found in some food stuffs, most notably as a preservative in cured meat and bacon.

The entero-salivary circulation of nitrate

In 1994, two groups independently described intragastric NO generation from salivary nitrite in humans[3,4]. This process does not require NOS activity, but instead involves the entero-salivary circulation of inorganic nitrate (FIG. 2). Dietary nitrate is rapidly absorbed in the upper gastrointestinal tract. In the blood, it mixes with the nitrate formed from the oxidation of endogenous NO produced from the NOS enzymes. After a meal rich in nitrate, the levels in plasma increase greatly and remain high for a prolonged period of time (plasma half-life of nitrate is 5–6 hours). The nitrite levels in plasma also increase after nitrate ingestion[8]. Although much of the nitrate is eventually excreted in the urine, up to 25% is actively taken up by the salivary glands and is concentrated up to 20-fold in saliva[8,40].

Once in the oral cavity, commensal facultative anaerobic bacteria use nitrate as an alternative electron acceptor to oxygen during respiration, effectively reducing salivary nitrate to nitrite by the action of nitrate reductases[7,38]. Human nitrate reduction requires the presence of these bacteria — suggesting a functional symbiosis relationship — as mammalian cells cannot effectively metabolize this anion. The salivary nitrate levels can approach 10 mM and nitrite levels 1–2 mM after a dietary nitrate load[8]. When saliva enters the acidic stomach (1–1.5 l per day), much of the nitrite is rapidly protonated to form nitrous acid (HNO_2; pKa ~3.3), which decomposes further to form NO and other nitrogen oxides[3,4]. Nitrite reduction to NO is greatly enhanced by reducing compounds such as vitamin C and polyphenols, both of which are abundant in the diet[17,18,41]. The importance of oral bacteria in gastric NO generation is perhaps most clearly illustrated in experiments using germ-free sterile rats, in which gastric NO formation is negligible even after a dietary load of nitrate[42]. In addition to the

Hypoxic vasodilation
A physiological phenomenon in which blood vessels dilate in response to low oxygen levels.

stomach, a reductive pathway from nitrate to nitrite and then NO has also been demonstrated in the oral cavity[7], on the skin surface[43] in the lower gastrointestinal tract[44] and in urine[45].

While the scientific community had focused on the potentially harmful effects of nitrate and nitrite[1], the well-known antibacterial effects of NO[46–49] suggested a role for gastric NO in host defence[3,4] (FIG. 3). Interestingly, enteropathogens can survive for a surprisingly long time in acid alone, but the combination of acid and nitrite results in effective killing[3,50,51]. NO and other reactive nitrogen oxides formed from acidified nitrite act on multiple bacterial targets including DNA, proteins and components of the cell wall[38,47].

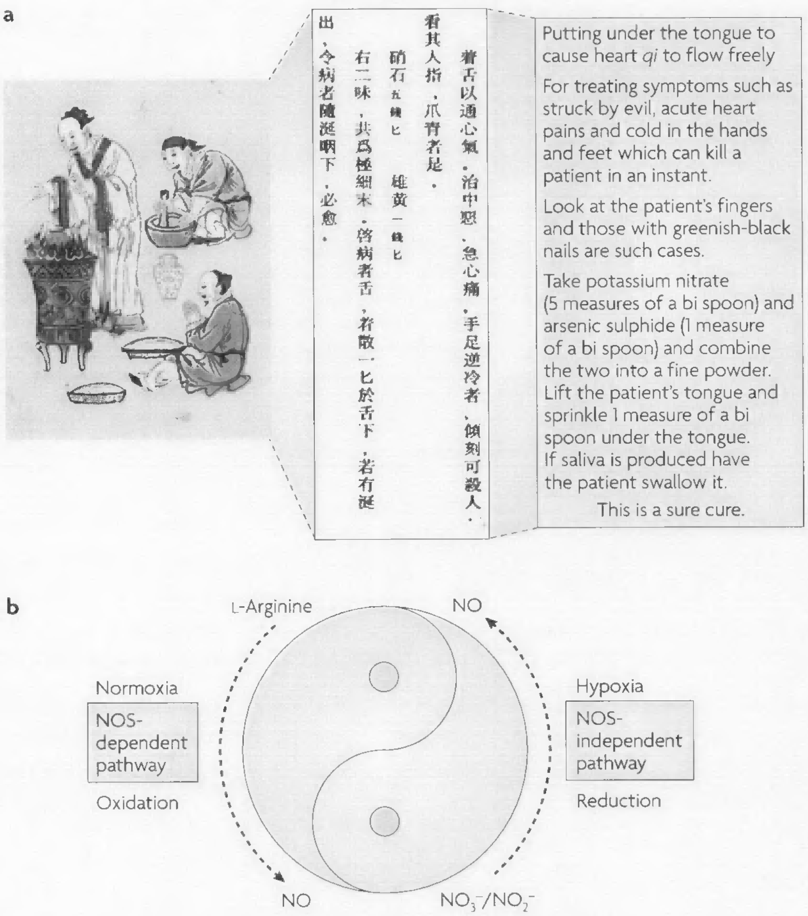

Figure 1 | **The nitrate–nitrite–NO pathway. a** | A medical recipe from Dunhuang. The nitrate–nitrite–nitric oxide (NO) pathway has been harnessed therapeutically since the medieval times as evidenced by a translation of medieval Buddhist manuscripts, which was discovered in a Buddhist grotto near the town of Dunhuang by a Daoist monk (Abbot Wang) at the beginning of the twentieth century after being hidden for 900 years. This was brought to our attention and translated by Anthony Butler, Zhou Wuzong and John Moffett. It illustrates the early appreciation of the effect of nitrate, readily available for meat-curing and gunpowder and reduced to nitrite in saliva, on cardiovascular conditions (angina and digital ischaemia). The text is written vertically beginning on the right and progressing leftwards. The term qi refers to a 'fluid' that, in a healthy person, flows harmoniously around the body. Its flow is disrupted during sickness. A bi spoon was a ceremonial spoon used in medicine. Chinese physicians often added realgar to a recipe as its colour is that of healthy blood. It would have had no effect because of its low solubility. **b** | Two parallel pathways for the generation of bioactive NO in mammals. NO is a key signalling molecule that serves to regulate a wide range of physiological functions. It is classically produced from L-arginine and oxygen by a family of enzymes, the NO synthases (NOSs). More recently, a fundamentally different mechanism for the generation of NO in mammals has been described. In this pathway, the inorganic anions nitrate and nitrite are reduced to form bioactive NO in blood and tissues during physiological hypoxia. Although NO generation by NOS becomes limited as oxygen levels fall, the nitrate–nitrite–NO pathway is enhanced. By the parallel action of both of these pathways, sufficient NO generation is ensured along the physiological and pathological oxygen and proton gradients.

Another proposed physiological role for gastric NO is in the regulation of mucosal blood flow and mucus generation. Recent studies using the rat gastric mucosa as an *in vivo* bioassay tested the effects of human saliva on these two important determinants of gastric integrity. When the rat gastric mucosa was exposed to human nitrite-rich saliva, NO gas was immediately generated and both mucosal blood flow and mucus thickness increased in a cyclic GMP-dependent manner[52]. Furthermore, nitrate addition to drinking water for 1 week produces similar effects[53]. During the nitrate treatment, nitrite accumulates in the gastric mucus and when this mucus is removed, the blood flow immediately returns to basal levels, indicating a continuous slow release of 'NO-like' bioactivity from nitrite trapped in the mucus[53]. A role for salivary nitrite in regulating gastric gastrin release has also been suggested[54].

Vasodilatory effects of nitrite

Although the vasodilatory properties of pharmacological doses of exogenous nitrite have been known for more than half a century[55–57], a physiological role of this anion in vasoregulation has been dismissed, even in more recent studies[58]. However, artery-to-vein gradients in nitrite across the human forearm, with increased consumption during exercise stress, suggests that nitrite is metabolized across the peripheral circulation[25]. Furthermore, humans breathing NO gas exhibit increases in peripheral forearm blood flow that is associated with increases in plasma nitrite[59], suggesting that nitrite could be a stable endocrine carrier and transducer of NO-like bioactivity within the circulation[60]. Consistent with a potentially greater efficacy under hypoxic or metabolic stress, the potency of nitrite increases dramatically with decreases in buffer pH in aortic ring experiments[24]. This hypothesis was tested by infusion of sodium nitrite into the forearm brachial artery of healthy volunteers, which was surprisingly potent, increasing blood flow even at blood concentrations below 1 μM and producing substantial vasodilation[6]. More recent dose-response experiments in normal human volunteers reveal significant vasodilation of the forearm circulation already at concentrations as low as 300 nM[61] and a significant decrease in blood pressure after nitrate ingestion, associated with an increase in plasma nitrite levels from 140–220 nM[62].

Pathways for systemic nitrite reduction

Haemoglobin as an allosterically regulated nitrite reductase. The ability of nitrite to vasodilate the circulation in the presence of NO-scavenging red blood cells under physiological conditions is unexpected and suggests new pathways to intravascular bioactivation. During infusion of nitrite into the human forearm circulation, the vasodilatory effects are associated with

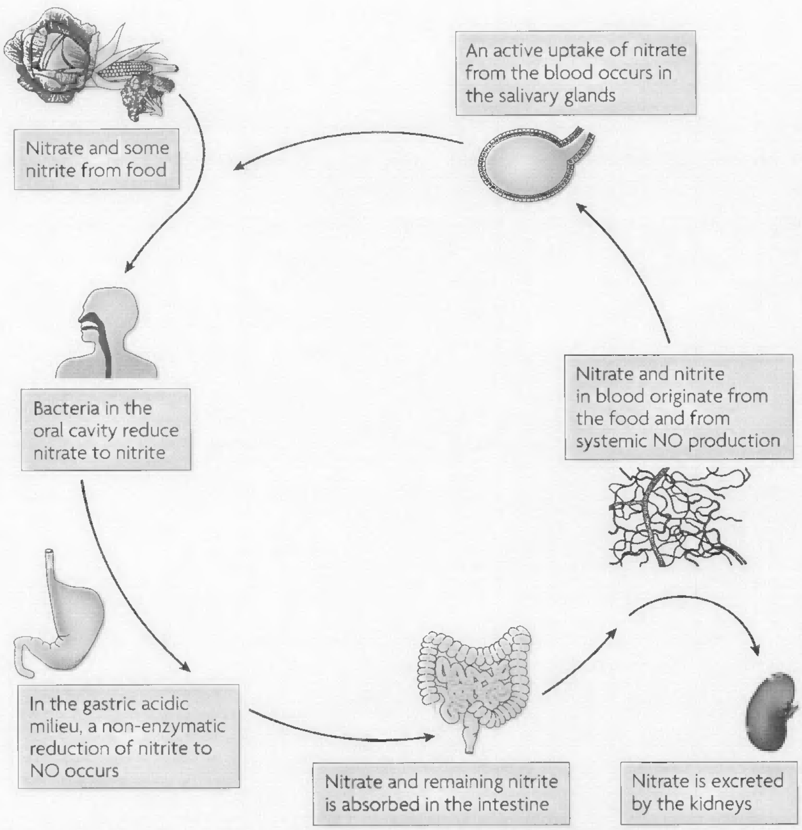

Figure 2 | **The entero-salivary circulation of nitrate in humans.** Ingested inorganic nitrate from dietary sources is rapidly absorbed in the small intestine. Although much of the circulating nitrate is eventually excreted in the urine, up to 25% is actively extracted by the salivary glands and concentrated in saliva. In the mouth, commensal facultative anaerobic bacteria effectively reduce nitrate to nitrite by the action of nitrate reductase enzymes. Nitrate reduction to nitrite requires the presence of these bacteria, as mammalian cells cannot effectively metabolize this anion. In the acidic stomach, nitrite is spontaneously decomposed to form nitric oxide (NO) and other bioactive nitrogen oxides, which regulate important physiological functions. Nitrate and remaining nitrite is absorbed from the intestine into the circulation and can convert to bioactive NO in blood and tissues under physiological hypoxia.

Methaemoglobin
A form of the oxygen-carrying protein haemoglobin in which the iron in the haem group is in the Fe^{3+} state, not the Fe^{2+} of normal haemoglobin. Methaemoglobin is unable to carry oxygen.

Facultative anaerobic bacteria
A bacterium, that makes ATP by aerobic respiration if oxygen is present but is also capable of switching to anaerobic respiration.

Electron acceptor
A chemical entity that accepts electrons transferred to it from another compound. It is an oxidizing agent that, by virtue of it accepting electrons, is itself reduced in the process.

the formation of NO in the blood, as measured by the rate of formation of iron-nitrosylated haemoglobin (NO bound to the haem of haemoglobin) during artery to vein transit[6]. This rate of NO formation increases as haemoglobin oxygen saturation decreases, suggesting a hypoxia-regulated mechanism of nitrite bioactivation.

These physiological findings are consistent with a nitrite reductase activity of deoxyhaemoglobin as described by Brooks in 1947 and Doyle and colleagues in 1981 (REFS 63,64). According to this chemistry, nitrite reacts with ferrous deoxyhemoglobin ($HbFe^{2+}$) and a proton (H^+) to generate NO and methaemoglobin ($HbFe^{3+}$), which is analogous to the coupled proton and electron-transfer reactions of bacterial nitrite reductases. The NO can then bind to a second deoxyhaemoglobin to form iron-nitrosyl-haemoglobin ($HbFe^{2+}$–NO) as outlined in equations 1 and 2.

$$\text{Nitrite } (NO_2^-) + \text{deoxyhaemoglobin } (Fe^{2+}) + H^+ \rightarrow$$
$$NO + \text{methaemoglobin } (Fe^{3+}) + OH^- \quad (1)$$
$$NO + \text{deoxyhaemoglobin } (Fe^{2+}) \rightarrow HbFe^{2+}\text{-}NO \quad (2)$$

This simple reaction has physiological implications in that it uses naturally occurring nitrite as a substrate, requires deoxygenation of haemoglobin so it has hypoxic sensor properties, requires a proton so it has pH sensor properties, and generates NO, the most potent vasodilator known. These chemical properties, and supporting physiological studies, suggest that haemoglobin may function as an allosterically regulated nitrite reductase that may contribute to hypoxic signalling and hypoxic vasodilation[6,10,65,66] (FIG. 3). The chemistry of this reaction, mechanisms of NO export from the red blood cell and physiological contribution to hypoxic blood flow regulation are the subjects of active research (FIG. 4). Interestingly, recent studies suggest that NO formed from nitrite reduction (equation 1) can react with a second nitrite that is bound to methaemoglobin ($HbFe^{3+}$)[67]. Remarkably, when nitrite binds to methaemoglobin, it forms a nitrogen dioxide radical ($^{\bullet}NO_2$) character ($HbFe^{3+}$–NO_2^- forms $HbFe^{2+}$–$^{\bullet}NO_2$), which reacts with NO in a radical–radical reaction to form N_2O_3. N_2O_3 is more stable in a haem-rich environment than NO and has the potential to escape from the red blood cell. The overall stoichiometry of the reaction of equation 1 and the second reaction of NO with nitrite–methaemoglobin is shown in equation 3. Note that in this reaction haemoglobin is catalytic and redox cycles convert two molecules of nitrite into N_2O_3.

$$2\,NO_2^- + \text{deoxyhemoglobin } (Fe^{2+}) + H^+ \rightarrow N_2O_3 + \text{deoxyhaemoglobin } (Fe^{2+}) + OH^- \quad (3)$$

Myoglobin, xanthine oxidoreductase and other pathways. Myoglobin has a high affinity for oxygen and a low haem redox potential that contributes to rapid nitrite reduction to NO when deoxygenated; in fact, deoxymyoglobin will reduce nitrite to NO at a rate 30-times faster than haemoglobin[11,66]. These chemical properties suggest that when myoglobin becomes deoxygenated, such as in the subendocardium of the heart or in exercising skeletal muscle, it will rapidly convert nitrite to NO (via the same nitrite reductase reaction as described above). Indeed, myoglobin has recently been shown to convert nitrite to NO in the cardiomyocyte and in the working heart[11,12]. NO formed by myoglobin can bind to cytochrome *c* oxidase of the mitochondrial electron transport chain, reducing electron flow and oxygen utilization[11]. Consistent with these studies, nitrite reduction to NO and nitrite-dependent modulation of cardiac consumption is abolished in the myoglobin knockout mouse[12]. These studies suggest that nitrite and myoglobin play an important role in regulating cardiac energetics and oxygen utilization under conditions of physiological hypoxia. Supporting this possibility, Larsen and colleagues found that whole-body oxygen consumption in young healthy volunteers was significantly reduced during submaximal exercise after dietary supplementation with nitrate compared with placebo treatment[68]. This surprising effect was associated with the metabolism of plasma nitrite.

Several enzymes, including xanthine oxidoreductase[13–15,69], complexes of the mitochondrial electron transport chain[70–72], cytochrome P450s[73] and even the

NOS enzyme[74], have been shown to use nitrite as an alternative electron acceptor to molecular oxygen thereby forming NO (FIG. 3). For example, xanthine oxidoreductase is known to reduce molecular oxygen to superoxide (O_2^-), but at low oxygen tensions and pH values this enzyme can also reduce nitrite to NO at the molybdenum site of the enzyme. In terms of a potential role in vasoregulation and NO signalling, these four pathways all require low oxygen tensions to effectively generate NO and these enzymes also produce superoxide, which is expected to react rapidly with and scavenge any NO that is synthesized. It is likely that nitrite can competitively reduce vascular reactive oxygen species (ROS) formation by these enzymes, by direct diversion of electrons away from oxygen, thus limiting superoxide formation. Decreases in superoxide will effectively

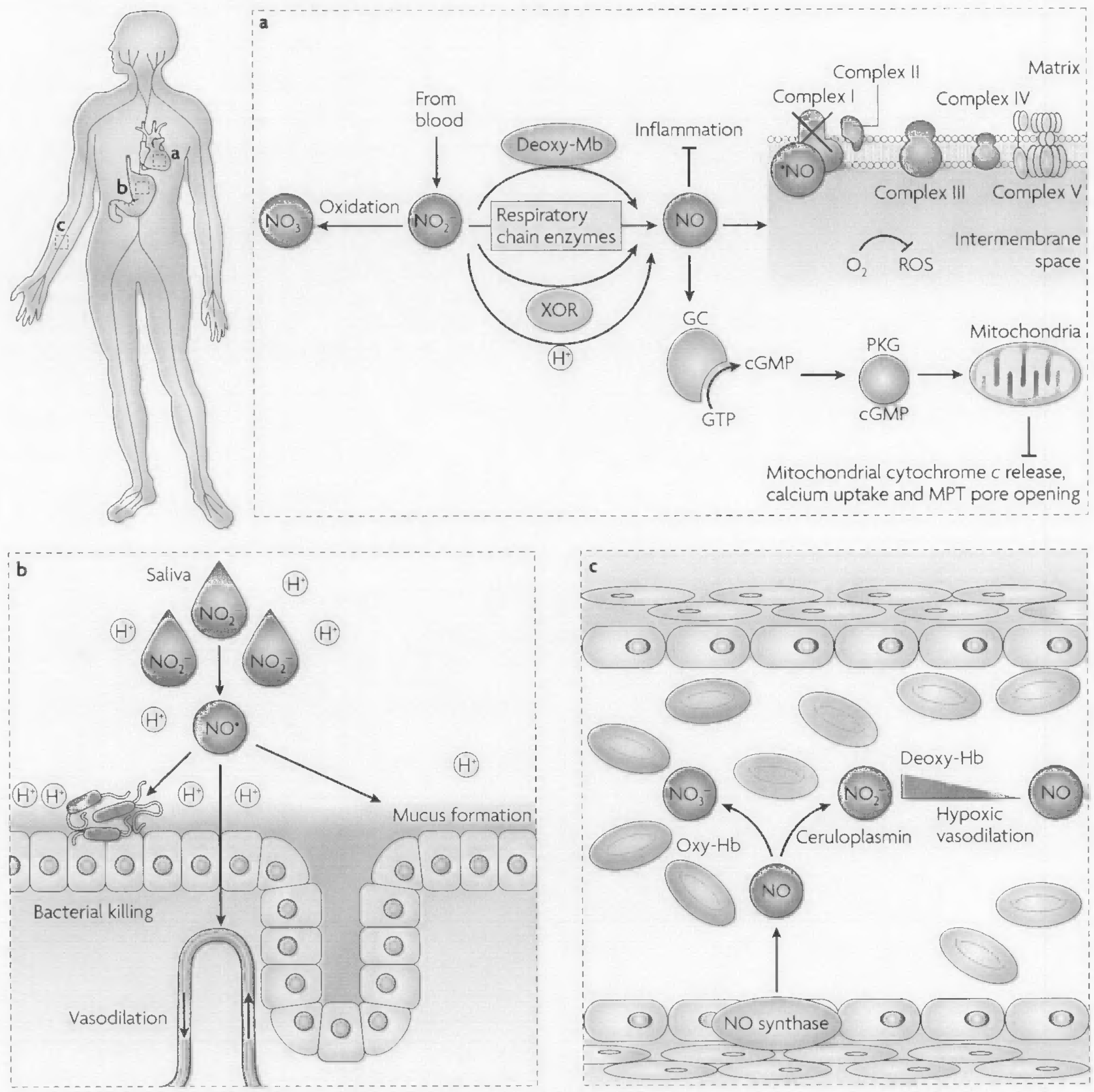

Figure 3 | **Pathways for nitrite reduction to NO and its proposed physiological roles. a** | In the tissues, such as the heart, there are numerous pathways for the generation of NO from nitrite, all greatly potentiated during hypoxia, including xanthine oxidoreductase (XOR), deoxygenated myoglobin (deoxy-Mb), enzymes of the mitochondrial chain and protons. Nitrite-dependent NO formation and S-nitrosothiol formation can modulate inflammation, inhibit mitochondrial respiration and mitochondrial derived reactive oxygen species formation, and drive cyclic GMP-dependent signalling under anoxia. NO-dependent cytochrome c oxidase (complex IV) inhibition can also drive reactive oxygen species (ROS)-dependent signalling. **b** | The formation of bioactive nitric oxide (NO) from the inorganic anion nitrite is generally enhanced under acidic and reducing conditions. In the acidic gastric lumen, NO is generated non-enzymatically from nitrite in saliva after formation of nitrous acid (HNO_2) and then decomposition into NO and other reactive nitrogen oxides. This NO helps to kill pathogenic bacteria and it also stimulates mucosal blood flow and mucus generation, thereby enhancing gastric protection. Detrimental effects have also been suggested, including nitrite-dependent generation of nitrosamines with potentially carcinogenic effects. **c** | In the blood vessels, nitrite forms vasodilatory NO after a proposed reaction with deoxygenated haemoglobin (deoxy-Hb) and contributes to physiological hypoxic blood flow regulation. GC, guanylate cyclase; MPT, mitochondrial permeability transition; Oxy-Hb, oxygenated haemoglobin; PKG, protein kinase G.

cyclic GMP
A cyclic nucleotide derived from guanosine triphosphate (GTP) that acts as a second messenger, much like cyclic AMP.

Allosteric
Allosteric regulation is the regulation of an enzyme or protein by binding an effector molecule at a site other than the protein's active site.

Mitochondrial electron transport chain
An electron transport chain associates energy-rich electron donors (for example, NADH) and mediates the biochemical reactions that produce ATP, which is the energy currency of life.

increase vascular NO bioavailabilty. In addition to secondary NO generation, a role of nitrite as an intrinsic signalling molecule that can directly modify target haem or thiol groups on proteins has been proposed[23].

Therapeutic opportunities

Vasodilation. Numerous studies have now confirmed the vasodilating effects of low-dose nitrite in mice, rats, sheep, dogs, primates and humans[75–81]. Therapeutic delivery of nitrite to vasodilate ischaemic vascular beds shows great promise in preclinical studies (FIG. 5). Patients suffering from spontaneous haemorrhage of a subarchnoidal artery aneurism are at risk for developing delayed cerebral artery spasm. In primate models, this spasm is associated with acute depletion of cerebral spinal fluid nitrite levels. Two-week infusions of systemic nitrite effectively prevented this complication[80].

Primary pulmonary hypertension of the newborn (PPHN) is a condition that is associated with a high pulmonary vascular resistance and extremely low systemic oxygenation. In sheep models of PPHN, inhaled nitrite was converted to NO gas in the lung and selectively vasodilated the pulmonary circulation[78]. In such diseases, which are characterized by regional ischaemia and vasoconstriction, nitrite may provide an ideal stable and naturally occurring therapeutic NO donor.

The vasodilatory and biological activities of the inorganic anions nitrite and nitrate must be distinguished from the organic nitrates (that is, nitroglycerin) and nitrites (amyl-nitrite). Clearly, the organic nitrates and nitrites are much more potent than nitrite in terms of vasoactivity. Although the anti-anginal and vasodilatory organic nitrates and nitrites are metabolized *in vivo* into vasodilatory NO and nitrite[82], this bioactivation requires metabolism by mitochondrial aldehyde dehydrogenase and other enzymes, which are all subject to induced tolerance[83,84]. Tolerance is characterized by a lack of nitroglycerin biological activity with chronic drug exposure. Studies from as far back as 1930 suggest that inorganic nitrite does not induce tolerance[85], implying that nitrite may represent an active metabolite of nitroglycerin that can bypass enzymatic nitroglycerin metabolism and tolerance.

Tissue protection in ischaemia–reperfusion injury. Systemic NOS-independent NO formation from nitrite was first demonstrated in the ischaemic heart[5]. Studies in animal models of ischaemia and reperfusion have now revealed a central role of nitrite in hypoxic signalling. Physiological and therapeutic levels of nitrite exert potent cytoprotection after prolonged ischaemia and blood-flow reperfusion in liver[22,86], heart[22,79,87], brain[88] and kidney[89]. These findings suggest an opportunity for nitrite therapy for human diseases associated with ischaemia–reperfusion, such as myocardial infarction, stroke, solid-organ transplantation, cardiopulmonary arrest and sickle-cell disease (FIG. 5).

Dose-response studies in mice suggest a broad efficacy to safety range of nitrite of three orders of magnitude, with doses as low as 0.1 μmoles per kg to 100 μmoles per kg providing significant protection. Interestingly, the protective effect of nitrite is evident at very low plasma concentrations (less than 200 nM), but is lost as plasma concentrations rise above 100–1,000 μM[22]. The lowest dose of nitrite given in these studies only increased the plasma levels of nitrite by 20%. Intriguingly, a similar or even greater increase in plasma nitrite is seen after ingesting a portion of spinach or lettuce[8]; this evokes provocative questions about a putative role of nitrate as an active ingredient of the cardioprotective mediterranean diet[9,38,90,91] (BOX 2).

The mechanism of nitrite-mediated cytoprotection appears to be NO-dependent and mitochondria-targeted. Studies using various inhibitors and genetic knockout mice provide some clues to the potentially important pathways. All the published animal studies have demonstrated a loss of cytoprotection when animals were treated with the NO scavenger carboxy-PTIO (2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide)[22,79,86,88], suggesting the importance of NO in the mechanism of cytoprotection. Pretreatment of animals with a NOS inhibitor[22,79] or use of eNOS knockout mice[22] did not inhibit cytoprotection, proving that the nitrite effect is NOS-independent.

The pathway(s) by which nitrite forms NO in hypoxic tissue remains to be determined. Two groups suggest the involvement of xanthine oxidoreductase in the reduction of nitrite to NO on the basis of reduced efficacy after treatments with allopurinol, a xanthine oxidase inhibitor[79,87,89]. The fact that nitrite remains protective in isolated buffer-perfused organ models, such as the Langendorff heart, suggests that the haemoglobin pathway is not necessary for this function. We have considered the possibility that in the heart, myoglobin can serve this function and have recently demonstrated that deoxymyoglobin has nitrite reductase activity, which can modulate mitochondrial respiration[11,21].

ROS generation by mitochondria is a necessary component of mitochondrial signalling in cytoprotection[92–95]. However, the large burst of oxidizing ROS generated after reperfusion following ischaemia can also contribute to cellular injury, necrosis and apoptosis[96,97]. *S*-Nitrosation of complex I of the electron transport chain inhibits the activity of this complex[98] and decreases mitochondrial-derived ROS formation during reperfusion, an effect associated with cellular cytoprotection[99,100]. Nitrite can similarly nitrosate complex I during ischaemia and reperfusion[21]. This modification limits complex I-dependent reperfusion ROS formation, activation of the mitochondrial permeability transition pore, and cytochrome *c* release. Interestingly, the effects of nitrite on mitochondria and tissue cytoprotection occur both acutely (immediately before reperfusion) and remotely (if given 24 hours before reperfusion), suggesting a potential role for nitrite as an effector of ischaemic preconditioning[21].

The inhibitory effect of NO[101–104] and nitrite on mitochondrial respiration that is associated with mitochondrial-dependent cytoprotection presents an interesting paradox. The energetic cost of reversibly inhibiting mitochondrial respiration appears to be offset by reduced ROS generation during reperfusion. A paradigm is emerging that damping electron flow to

Reactive oxygen species
(ROS). Include oxygen ions, free radicals and peroxides that are both inorganic and organic. They are generally highly reactive owing to the presence of unpaired valence shell electrons. ROS form as a natural by-product of the normal metabolism of oxygen and have important roles in cell signalling. However, during times of environmental stress ROS levels can increase dramatically, which can result in significant damage to cell structures.

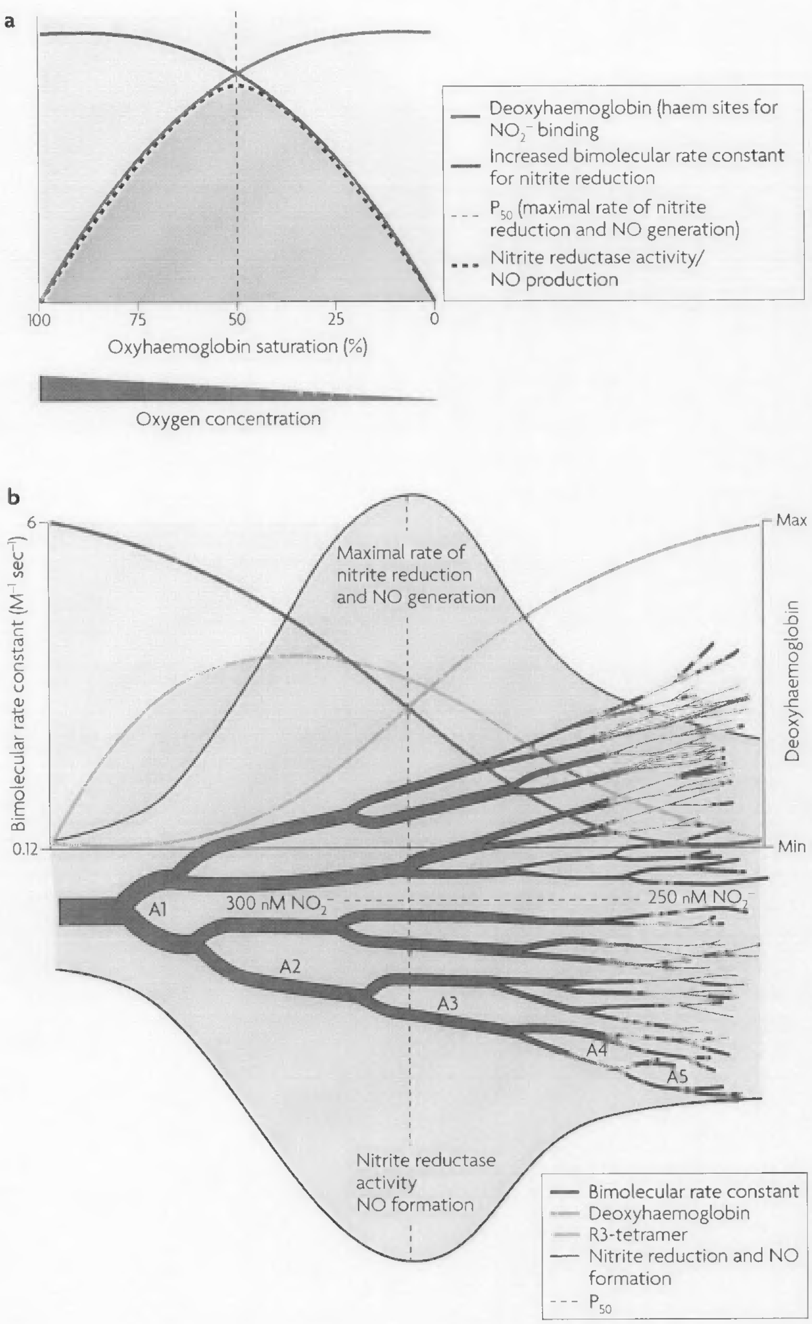

Figure 4 | **R-state or allosteric autocatalysis of nitrite reduction by haemoglobin. a** | Allosteric nitrite reduction by haemoglobin. The rate at which haemoglobin converts nitrite into nitric oxide (NO) is maximal when it is 50% saturated with oxygen, the midpoint of the haemoglobin–oxygen dissociation curve (P_{50}). This effect is mechanistically determined by two opposing chemistries, the availability of deoxyhaems (reaction substrate) to bind nitrite, which is maximal in T-state or deoxygenated haemoglobin, and the amount of R-state or oxygenated haemoglobin tetramer, which increases the intrinsic reactivity of the haem with nitrite. The latter process occurs because R-state or oxygenated haemoglobin has a decreased haem redox potential, which is analogous to the low haem redox potential of myoglobin. This low redox potential of the R-state ferrous haem favours an equilibrium distribution of electrons to nitrite, resulting in the increased reactivity of nitrite with the unliganded ferrous haems of R-state haemoglobin and myoglobin. The lowered haem redox potential is kinetically manifested as an increased bimolecular rate constant for the reaction of nitrite with R-state haemoglobin compared with T-state haemoglobin. Because the observed rate of nitrite reduction to form NO is equal to the product of the bimolecular rate constant times the deoxyhaem (reactant) concentration, this rate is expected to be maximal at approximately 50% oxygen saturation, or the intrinsic haemoglobin P_{50}; this allows for physiological hypoxic 'sensing' and NO generation. **b** | Physiological model. In the mammalian circulation, the allosteric state of haemoglobin tetramers is modulated primarily by oxygen ligation such that the *in vivo* intrinsic reactivity (bimolecular rate constant) of the deoxyhaem is therefore dictated by oxygen binding to other haems on the same tetramer. This model therefore predicts that the most effective tetrameric nitrite reductase would be the R-state haemoglobin that rapidly deoxygenates during arterial to capillary transit. This molecule would transition through R4 (R-state with four oxygens bound) to R3 (R-state with three oxygens bound) to R2 intermediates and then shift to T2 (T-state with two oxygens bound) and ultimately T1. These R3 and R2 tetramers would be the most effective nitrite reductases (bimolecular rate of 6 M^{-1} sec^{-1} for R-haem compared with 0.03 M^{-1} sec^{-1} for T-haem). This chemistry is consistent with experiments showing that nitrite reduction and NO signalling are most effective in systems subjected to rapid deoxygenation in the presence of oxyhaemoglobin and nitrite or when haemoglobin is 50% saturated with oxygen. A1–A5 reflect the arteriolar size, which decreases at branch points.

Organic nitrates
Drugs used principally in the treatment of angina pectoris and acting mainly by dilating the blood vessels by the formation of nitric oxide.

***S*-nitrosation**
The conversion of thiol groups (-SH), including cysteine residues in proteins, to form *S*-nitrosothiols. *S*-Nitrosation has been suggested to be a mechanism for dynamic, post-translational regulation of proteins. In addition, *S*-nitrosothiols can act as

oxygen (thus limiting superoxide formation) during reperfusion, by NO-dependent complex I and IV inhibition[98,101] or by depleting oxygen during reperfusion (post-conditioning), may reduce reperfusion ROS generation and limit downstream apoptotic signalling[105]. Data also suggest that NO-dependent inhibition of cytochrome *c* oxidase before ischaemia, that is, during normoxia, can produce the opposite effect of increasing basal ROS formation, creating a preconditioning environment that is also adaptive[92–95].

Several recent studies of NO gas inhalation in both animals and humans suggest a transformation of NO in the lung into a more long-lived bioactive NO-species that can be transported in blood[59,106]. Moreover, inhaled NO reduces myocardial infarction volume in mice[107] and pigs[108] and the extent of liver injury after orthotopic transplantation in humans[109]. These effects are associated with significant increases in circulating nitrite, with no significant changes in blood *S*-nitrosothiol levels. NO treatment significantly reduced the overall incidence of brain injury in premature newborns with respiratory failure, an effect consistent with endocrine transport of an NO-intermediate in blood to the central nervous system[110]. Thus, increasing evidence suggests that nitrite is mediating extrapulmonary effects of NO gas inhalation.

The promising animal data discussed here indicate that nitrite possesses the characteristics of a useful adjunctive therapy for acute myocardial infarction, including

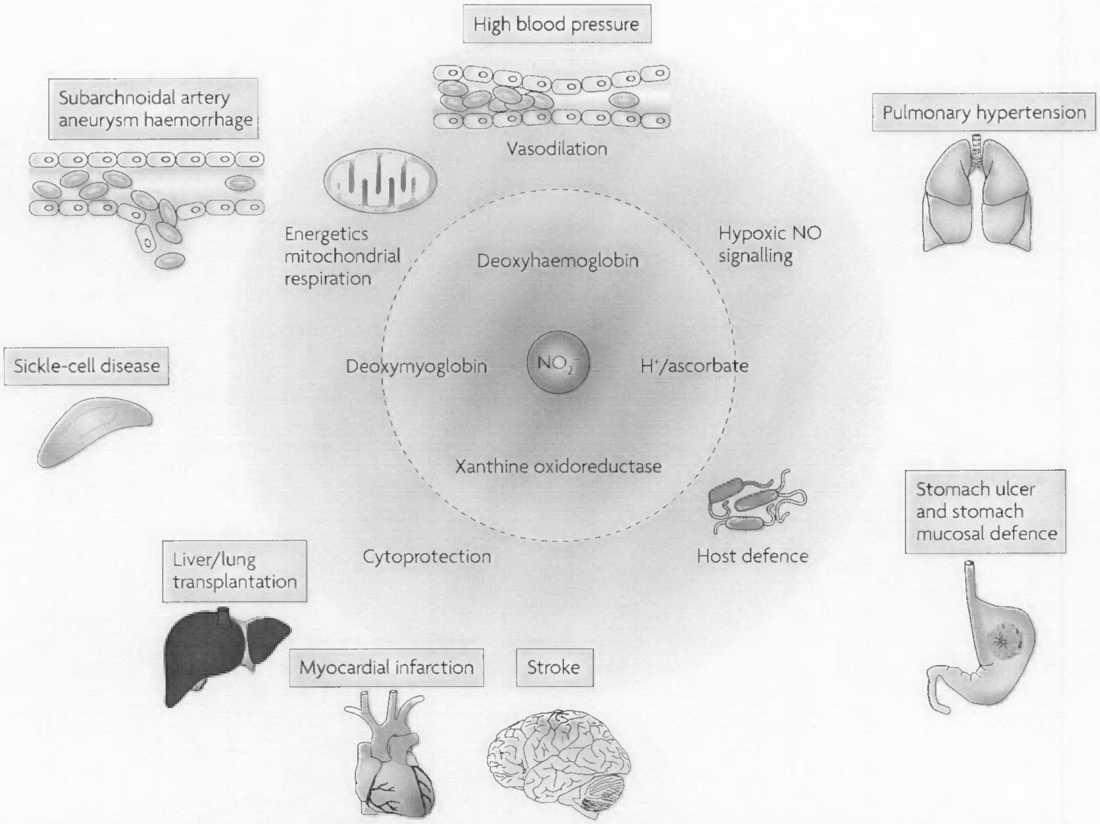

Figure 5 | **Therapeutic opportunities for inorganic nitrite.** The inorganic anion nitrite (NO_2^-) can be metabolized in blood and tissues to form nitric oxide (NO), a pluripotent biological messenger. Nitrite reduction to NO is catalysed by various enzymatic and non-enzymatic pathways and is greatly enhanced during hypoxia and ischaemic stress, which may be of therapeutic value. In animal models, nitrite is strongly cytoprotective and protects against ischaemia–reperfusion injury. These findings suggest an opportunity for nitrite therapy for human diseases such as myocardial infarction, stroke, solid-organ transplantation and sickle-cell disease. Nitrite is also cytoprotective in the stomach, where it can prevent drug-induced gastric ulcers. The vasodilatory and blood-pressure lowering effects of nitrite could be useful in pulmonary and systemic hypertension, as well as in the treatment and prevention of delayed cerebral vasospasm after subarachnoidal artery aneurysm haemorrhage.

significant cardioprotection following prolonged ischaemia, simple administration and minimum associated regional and systemic side effects. Based on these considerations, a human Phase II clinical trial of intravenous nitrite for ST segment elevation myocardial infarction is currently being planned by the US National Heart, Lung, and Blood Institute in cooperation with European centres.

Gastric ulcers. A common and potentially serious side effect of aspirin-like drugs (non-steroidal anti-inflammatory drugs; NSAIDs) is the development of gastric ulcers secondary to the inhibition of prostaglandin synthesis by these agents[111]. Similarly, in animal models, pharmacological inhibition of the NOS enzymes increases the susceptibility to ulcerogenic compounds[112]. Experiments with isoform-selective inhibitors suggest that the constitutive isoforms of cyclooxygenase (COX1) and NOS (eNOS and neuronal NOS; nNOS) are protective, while the opposite may be true for the inducible enzymes (COX2 and iNOS)[113,114]. In a recent study, rats were given sodium nitrate in the drinking water for 1 week followed by acute exposure to an NSAID (diclofenac) by gastric gavage[115]. Dietary nitrate increased gastric NO levels and potently protected against the macroscopic injury caused by NSAID exposure (FIG. 5). Additionally, nitrate pretreatment decreased mucosal myeloperoxidase activity and expression of iNOS, which is indicative of reduced tissue inflammation. The protection afforded by nitrate probably relates to increased gastric mucosal blood flow and mucus generation and reduced epithelial permeability[52,53].

The gastroprotective effect of nitrate was abolished in rats if they were pretreated with topical antibiotics in the mouth before nitrate supplementation, thereby illustrating the importance of the oral microflora in the bioactivation of nitrate[116]. An additional protective effect of nitrate on ulcer development may occur through inhibition of *Helicobacter pylori*[117].

In critically ill patients, endotracheal intubation and sedation interrupt the entero-salivary nitrate cycle, which results in depleted gastric NO, nitrite and *S*-nitrosothiol levels[118]. It has been suggested that the insufficient levels of gastric NO contribute to the gastric lesions and bacterial overgrowth commonly found in these patients[118].

Box 2 | Dietary aspects of nitrate and nitrite

The metabolism of dietary nitrate can result in intragastric formation of nitrosamines, which may be carcinogenic[1]. However, despite more than 40 years of extensive research, there is still no clear evidence for a link between nitrate intake and gastric cancer in humans[133]. Moreover, it is well known that a diet rich in vegetables is associated with a lower blood pressure and a reduced long-term risk for the development of cardiovascular disease[134,135]. Recently, Larsen and colleagues[62] performed a double-blind placebo-controlled crossover evaluation of dietary nitrate supplementation in healthy young volunteers and found a significant reduction in resting blood pressure with a nitrate dose corresponding to the amount found in 150–250 g of a green leafy vegetable. Remarkably, the reduction in blood pressure was similar to that described in healthy controls consuming a diet rich in fruits and vegetables in the Dietary Approaches to Stop Hypertension (DASH) trial[136]. Consistent with this, new preclinical studies now indicate that dietary levels of nitrite and nitrate significantly modulate the susceptibility to cardiac and liver ischaemia–reperfusion injury and gastric and intestinal mucosal integrity, respectively[115,127]. In recent studies in rats and mice, oral intake of nitrite either immediately before or even 24 hours before myocardial infarction significantly reduced infarction volume[21]. In this context, nitrate may be considered as a 'prodrug', which produces a sustained delivery of nitrite to the systemic circulation following entero-salivary circulation[8]. The possibility of boosting nitric oxide production by dietary intervention may have important implications for public health, in particular cardiovascular disease. The central role of commensal bacteria in the bioactivation of nitrate is intriguing and suggests that a symbiotic host–microbial relationship is involved in the regulation of cardiovascular function.

Future clinical studies will elucidate whether nitrate can offer a nutritional approach to the prevention and treatment of disease. If such investigations point towards a protective effect of nitrate, the current strictly regulated levels of nitrate in food and drinking water may need to be reconsidered.

Antimicrobial effects. Nitrite is used as a preservative in meat products to inhibit the growth of pathogens, most notably *Clostridium botulinum*, and these antibacterial effects have been attributed to NO formation[119]. The discovery of endogenous nitrite reduction to NO in the acidic stomach triggered researchers to explore therapeutic uses for acidified nitrite as an antimicrobial agent. Indeed, acidified nitrite results in the generation of NO and other nitrogen oxides, which have potent antibacterial activity against a range of pathogens, including *Salmonella*, *Yersinia* and *Shigella* species, *H. pylori*, and *Pseudomonas aerguinosa*[3,16,117,120]. These antibacterial effects of nitrite have recently been investigated in the airways. In an animal model resembling cystic fibrosis, acidified nitrite successfully cleared the airways of mucoid *P. aeruginosa*, a pathogen commonly infecting the airways of patients with cystic fibrosis[121].

Infected urine typically contains considerable amounts of nitrite, owing to bacterial reduction of urinary nitrate. Although nitrite is stable at neutral or alkaline conditions, it is reduced to NO and has potent antibacterial effects if the urine is mildly acidified (to pH 5–6); these effects are potentiated in the presence of the reducing agent vitamin C[16]. In fact, the *in vitro* antibacterial potency of nitrite and ascorbic acid is fully comparable to that of traditional antibiotics such as nitrofurantoin and trimetoprim. Acidification of urine — for example, by vitamin C intake — has been used in traditional medicine for the prevention and treatment of urinary tract infections. This effect may be related to the formation of antibacterial nitrogen oxides from the acidified nitrite[38,122].

Opportunities for drug development

As mentioned above, several different therapeutic indications for nitrite have been successfully tested recently both in animal models and in humans (FIG. 5). Depending on the condition to be treated, the development of several different nitrite-containing formulations and methods of administration are anticipated.

Topical administration of acidified inorganic nitrite. An inorganic nitrite salt such as sodium nitrite ($NaNO_2$) is combined with an acidifying agent (for example, ascorbic acid). This mixture rapidly releases NO and other nitrogen oxides and has been evaluated for its antimicrobial activity. Topical application of acidified nitrite to the skin has proved effective in various skin infections[123–125], and in the airways, acidified nitrite has been shown to kill mucoid *Pseudomonas* in an animal model of cystic fibrosis[121]. Carlsson and colleagues used the inflatable retention balloon of a urinary catheter as a depot for nitrite and ascorbic acid, leading to direct intravesicular delivery of antimicrobial nitrogen intermediates[126]. In their *in vitro* studies, NO was generated in the retention balloon and diffused into the surrounding urine where it effectively killed the urinary pathogen *Escherichia coli*. They suggested that this could be a new approach to prevent catheter-associated urinary-tract infections, the most common hospital-acquired infection.

Enteral administration of inorganic nitrite and nitrate. It is clear that both nitrate and nitrite are readily absorbed and biologically active when given orally, and therapeutic effects have been observed in animal models of ischaemia–reperfusion injury[127] and in protection against gastric ulcerations[115,116,128]. In addition, short-term dietary nitrate supplementation has been shown to lower blood pressure in healthy volunteers[62]. A combination of nitrate and nitrite salts for oral administration is theoretically attractive, as the nitrite would ensure immediate effects soon after absorption, while the nitrate would continuously provide a slow release of nitrite over a prolonged period of time via the entero-salivary recirculation described above. Similar to the recently developed NO–NSAIDs, in which the active drug is combined with an organic nitrate[129], the addition of inorganic nitrate to an ulcerogenic drug such as aspirin or another NSAID is also a possible new composition.

Organic nitro compounds as donors of nitrite. The bioavailability of nitrite after enteral administration of inorganic nitrate or nitrite can be difficult to control because of the variable metabolism of these anions within the gastrointestinal tract. However, the use of organic allylic nitro compounds as nitrite donors may overcome this potential problem[130], as *in vitro* experiments have shown that such compounds can release nitrite and NO in the presence of thiols (L-cysteine) and ascorbic acid. Traditional organic nitrates (nitroglycerine) and nitrites (amyl nitrite) used in cardiovascular medicine are also metabolized to nitrite *in vivo*. Whether the organic allylic nitro compounds or other donors of nitrite can offer any additional advantages over these

compounds and the native inorganic anions, in terms of controlled delivery, bioavailability and tolerance, remains to be studied.

Short or long-term infusions of inorganic nitrite. In the development of nitrite for therapeutic intravenous use, it is anticipated that the dose and duration of treatment will have to be adjusted depending on the condition and the desired effect. In animal studies, large doses of nitrite infused over a long period of time are needed to effectively alleviate the vasospasm associated with subarachnoidal haemorrhage[80]. In models of ischaemia–reperfusion injury, however, the dose of nitrite needed for protective effects is remarkably low[21,22].

Toxicity. The two major health concerns with inorganic nitrite and nitrate are the risk for development of methaemoglobinaemia and their potential carcinogenic effects[2]. Any toxicity of the nitrate ion is thought to occur after its bioconversion to nitrite, which is considerably more reactive. Formation of methaemoglobin occurs when the oxygen-carrying ferrous ion (Fe^{2+}) of the haem group of the haemoglobin molecule is oxidized by nitrite to the ferric state (Fe^{3+}). This converts haemoglobin to methaemoglobin, which cannot bind oxygen. Clinically significant methaemoglobinaemia with cyanosis occurs when the levels increase above a certain level (approximately 5%). In animal studies looking at the tissue protective and vasodilatory effects of intravenous nitrite, the increase in methaemoglobin is generally undetectable or modest even after prolonged delivery[80], suggesting that methaemoglobin is not a major problem in these dose ranges. In fact, the estimated EC_{50} for nitrite in human adults, based on methaemoglobin formation, is 1 g[131], whereas calculations from animal data suggest that less than 40 mg nitrite would be necessary for the treatment of myocardial infarction in a 70 kg adult.

In 2001, the US Department of Health and Human Services National Toxicology Program published extensive toxicology and carcinogenesis studies of sodium nitrite in rats and mice. Sodium nitrite was delivered in drinking water for 14-week and 2-year periods and genetic toxicology studies were conducted in *Salmonella typhimurium*, and in rat and mouse bone marrow and peripheral blood. Consistent with recent epidemiological studies in humans[2], there was no significant evidence of carcinogenic activity of nitrite, despite dose escalations sufficient to produce profound methaemoglobinaemia and weight loss in rodents[131]. A recent epidemiological study evaluating dietary exposure of nitrite (cured meat) has suggested a possible link to the development of emphysema in at-risk subjects[132]; further population studies will be required to validate this observation. For most of the therapeutic indications discussed in this article, the low dose and short duration of treatment suggest that the risk of any carcinogenic effects is negligible. In fact as stated above, a large consumption of nitrate-containing vegetables may provide similar or even greater systemic loads of both nitrate and nitrite. If nitrite is to be used in much higher doses over prolonged periods of time, this issue will naturally have to be addressed.

Conclusions

The nitrate–nitrite–NO pathway may be viewed as complementary to the classical L-arginine–NOS pathway. These pathways work partly in parallel, but when oxygen availability is reduced and NOS activity is decreased, nitrite reduction to NO becomes more pronounced. So, in pathological conditions when regional and systemic ischaemia prevail, it may be beneficial to support the nitrate and nitrite stores pharmacologically or by dietary intervention.

We must now revise our long-standing view that nitrate and nitrite are only harmful substances in our diet or inert metabolites of endogenous NO. Instead, accumulating evidence suggests that the nitrate–nitrite–NO pathway critically subserves physiological hypoxic NO signalling, providing an opportunity for novel NO-based therapeutics.

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Acknowledgements
We wish to thank J. Moffett, librarian of the Needham Research Institute, University of Cambridge, UK, for providing the image used in figure 1a, and G. K. Hansson for valuable comments on the manuscript. We have received support from the European Community's Sixth Framework programme (Eicosanox, LSMH-CT-2004-005033), the Swedish Heart and Lung Foundation, The Swedish Research Council, Torsten and Ragnar Söderbergs Foundation, Vinnova (CIDaT), and Stockholm County Council (ALF).

Competing interests statement
The authors declare competing financial interests: see web version for details.

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Fd Chem. Toxic. Vol. 20, pp. 25 to 33, 1982
Printed in Great Britain.

0278-6915/82/010025-09$03.00/0

CARCINOGENICITY STUDIES OF SODIUM NITRITE AND SODIUM NITRATE IN F-344 RATS*

A. MAEKAWA, T. OGIU, H. ONODERA, K. FURUTA, C. MATSUOKA, Y. OHNO and the late S. ODASHIMA

Division of Pathology, Biological Safety Research Centre, National Institute of Hygienic Sciences, Kamiyooga 1-18-1, Setagaya-ku, Tokyo 158, Japan

(*Received 25 March 1981*)

Abstract—The carcinogenicity of sodium nitrite and of sodium nitrate was examined in F-344 rats. Sodium nitrite was administered in the drinking-water for 2 yr at levels of 0·125 or 0·25%. Sodium nitrate was given in the diet at levels of 2·5 or 5%. A variety of tumours occurred in all groups including the controls. The only significant difference between treated and control groups in the total number of tumours detected in either of the studies was a significant decrease in tumour incidence in the high-dose females given nitrite compared with controls. There was no positive dose-response relationship either in the incidence or in the induction time of tumours in either of the studies. The only significant result was a reduction in the incidence of mononuclear cell leukaemias in the experimental groups in both studies. It is concluded that sodium nitrite and sodium nitrate did not exert a carcinogenic effect that could be detected under the conditions of this study in which the animals showed a high incidence of spontaneous tumours.

INTRODUCTION

Sodium nitrite and sodium nitrate are widely used in Japan and throughout the world as food additives to preserve and colour cured meat and/or fish. It was recently reported that sodium nitrite has strong mutagenic activity in various mutagenicity tests (Odashima, 1980). It has also been demonstrated that sodium nitrite is a precursor of *N*-nitroso compounds, many of which have strong carcinogenic activity in many species of animals. Sodium nitrate is partially reduced to sodium nitrite both in the animal body and in food (Heisler, Siciliano, Krulick *et al.* 1974; Ishiwata, Boriboon, Nakamura *et al.* 1975). But until now there have been no significant reports on the carcinogenicity test of these two chemicals. The present studies were carried out to clarify the carcinogenicity of these two chemicals.

EXPERIMENTAL

Subchronic toxicity study

A total of 240 Fischer-344 rats (SPF, 5-wk-old) of both sexes were purchased from Charles River Japan Inc. (Kanagawa). Rats were housed four to a plastics cage and kept in an air-conditioned animal room (temperature 25 ± 2°C, humidity 55 ± 10%).

Sodium nitrite (special grade reagent, purity 98·5%) and sodium nitrate (special grade reagent, purity 99·5%) were purchased from Koso Chemical Co. Ltd (Tokyo). The stability of the sodium nitrite when mixed with the diet was found to be very low (13–27% recovery) and it was therefore administered in the drinking-water. A fresh solution of sodium nitrite in distilled water was prepared daily. Sodium nitrate was found to be stable in the diet (about 100% recovery) and was therefore mixed with the basic diet (CRF-1, Charles River Japan Inc.) which was then pelleted.

The animals were divided into groups of ten male and ten female rats. In the sodium nitrate study five experimental groups were given as drinking-water 20 ml of a solution of sodium nitrite/rat/day at concentrations of 1, 0·5, 0·25, 0·125 or 0·06% for 6 wk. Rats in the control group were each given 20 ml distilled water/day. In the sodium nitrate study the five treated groups were given 20, 10, 5, 2·5 or 1·25% sodium nitrate in the diet *ad lib.* for 6 wk. The control group was given basic diet only. In the nitrate study all groups were given tap water freely.

During the experimental periods, all the animals were observed daily; signs of toxicity and mortality were recorded, and body weights were determined every other week. Dead animals were completely autopsied. At the end of the study, all surviving animals were killed for gross and microscopical examinations. The results of this study were used to determine the appropriate dose-levels in the carcinogenicity study.

Carcinogenicity study

Rats. A total of 600 F-344 rats of both sexes (SPF, 5-wk-old), purchased from Charles River Japan Inc., were maintained on the basic diet (CRF-1) and tap water, until they were 8-wk-old when the studies were started. Animals were divided into groups comprising 50 male and 50 female rats.

Housing and feeding conditions. Rats were housed four males and five females to a plastics cage and kept in an air-conditioned animal room (conventional animal room without barrier system) at a temperature

*Part of this work was presented at the 39th Annual Meeting of the Japanese Cancer Association, Tokyo, November 1980. The work was supported by grants-in-aid for cancer research from the Ministry of Health and Welfare, Japan.

Abbreviations: NDMA = *N*-Nitrosodimethylamine: SPF = specific-pathogen free.

25 ± 2°C and a relative humidity of 55 ± 10%. The basic diet was analysed for contaminants twice a year and it was ascertained that contaminants such as pesticides, metals, benzopyrene and aflatoxin were not included in the diet. Sodium nitrite and sodium nitrate were as used in the subchronic toxicity study.

Experimental design. In the nitrite study, the experimental groups were given 20 ml 0·25% (maximum tolerated dose) or 0·125% sodium nitrite/rat/day as their drinking water for 2 yr. Rats in the control group were each given 20 ml distilled-water/day for 2 yr. In the nitrate study the experimental groups were given diets containing 5% (maximum tolerated dose) or 2·5% of sodium nitrate *ad lib.* for 2 yr. Rats in the control group were given basic diet without nitrate *ad lib.* for 2 yr. The stability of nitrate in the diet was determined whenever new diet was received, and it was ascertained that the recovery rate of the chemical was about 100% at all times. Administration of chemicals was stopped at wk 104 and thereafter tap water and basic diet was given in all experimental groups, and observation was continued until wk 120 in the nitrite study and wk 123 in the nitrate study when all survivors were killed. These times were selected because the number of survivors in at least one group of either sex was less than 10 (20%). During the experimental period all animals were observed daily, and clinical signs and mortality were recorded. The amount of nitrite-containing water consumed/day or the amount of nitrate-containing diet consumed/month was measured. Body weights were recorded once a week during the first 10 wk of the study and every 2 wk thereafter. Moribund or dead animals were autopsied completely and examined for the development of tumours in various organs and/or tissues. Organs and/or tissues were fixed with buffered 10% formalin, and sections were stained routinely with haematoxylin and eosin.

Analysis of nitrosamines. Analysis of nitrosamines in the basic diet, diet containing nitrate, drinking-water containing nitrite and the stomach contents of treated rats was carried out twice using a Thermo-Energy Analyser (TEA-502), Shimazu Inc., Japan). For analysis of *N*-nitrosamines in the stomach contents, groups of ten male and ten female 10-wk-old F-344 rats were given identical treatments to the animals in the main study. After 1 wk of treatment the animals were killed and their stomach contents were analysed.

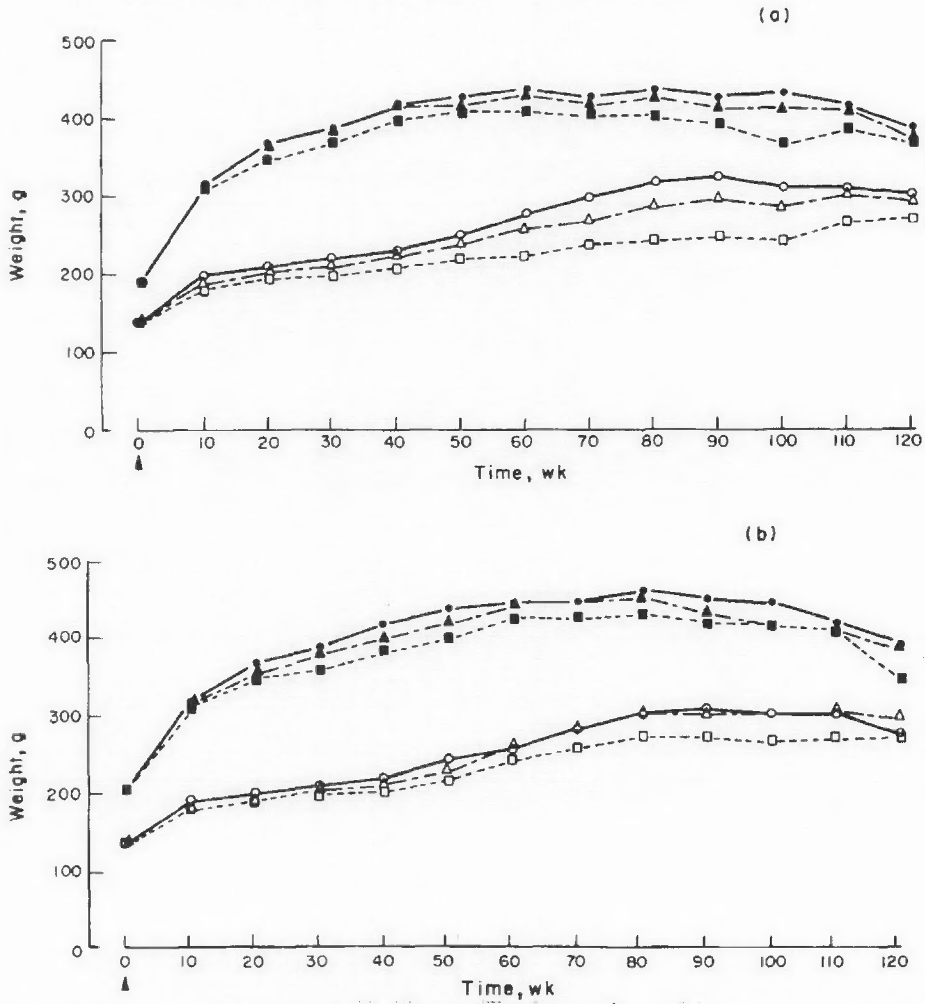

Fig. 1. Growth curves of rats treated with (a) sodium nitrite in the drinking water and (b) sodium nitrate in the diet. Control males (—●—), low-dose males (-—▲—-), high-dose males (---■---), control females (—○—), low-dose females (-—△—-) and high-dose females (---□---).

Table 1. *Cumulative mortality of rats treated with sodium nitrite in the drinking-water or sodium nitrate in the diet*

Sex	Dose†	Cumulative mortality (%) at 80 wk	100 wk	Term‡
		Sodium nitrite study		
M	0 (control)	16	32	76
	0·125	6	30	52*
	0·25	4*	12*	42**
F	0 (control)	2	14	54
	0·125	4	14	46
	0·25	8	16	48
		Sodium nitrate study		
M	0 (control)	6	20	80
	2·5	0	8	52**
	5·0	8	24	62*
F	0 (control)	12	36	72
	2·5	6	20	46**
	5·0	12	24	50*

†The dose is expressed as the percentage in the drinking-water (sodium nitrite) or diet (sodium nitrate).
‡In the sodium nitrite study, 120 wk; in the sodium nitrate study, 123 wk.
Values marked with asterisks differ significantly (chi-square test) from the corresponding control values (*$P < 0·05$; **$P < 0·01$).

RESULTS

Subchronic toxicity study

During the 6 wk of sodium nitrite treatment four female rats in the 1% group, and one male and one female in the 0·5% group died. None of the other treated or control rats died. In all the experimental groups except the 1% group, depression of body-weight gain compared with the control group was less than 10%. In the case of nitrate, all the females and seven males in the 20% group died during the experiment. In all groups except the males given 20% and the females given 10% nitrate, the decrease of body-weight gain compared with controls was less than 10%.

At autopsy the abnormal colour of the blood and the spleen due to methaemoglobin was marked in rats of the two highest dose groups in both studies.

From these results it was determined that the maximum tolerated doses of sodium nitrite and nitrate in F-344 rats were 0·25% in drinking-water and 5% in the diet, respectively.

Carcinogenicity studies of nitrite and nitrate

The first rats with tumours were autopsied at wk 56 in the sodium nitrite study and at wk 36 in nitrate study. Rats that survived beyond wk 56 and wk 36 in the nitrite and nitrate studies respectively were included in the data. Figure 1 shows the growth curve of the animals in both studies. In the male high-dose groups the mean body weight differed from that of the control group by less than 10% at all times in both studies, but in the female high-dose groups mean body weights were reduced by more than 10% after wk 40 in the nitrite study and after wk 60 in the nitrate study. Table 1 demonstrates the cumulative mortality of rats in the two studies. At term both male and female control groups contained the lowest numbers of survivors in both studies.

The amount of nitrite or nitrate consumed, the total tumour incidence including both benign and malignant tumours and the mean survival times are given in Table 2. Daily consumption of water or diet was almost constant throughout the experimental period in all groups. These consumption data were used to calculate the nitrite and nitrate intake data given in Table 2. The incidence of tumours was 100% in all male groups in the nitrite study and nearly 100% in all male groups in the nitrate study. However there was a reverse dose-effect relationship in the incidence of tumours in the females of both studies. The tumour incidence in the high-dose group was signifi-

Table 2. *Amount of nitrite or nitrate consumed, tumour incidence and mean survival time of F-344 rats after continuous oral administration of sodium nitrite or nitrate*

Dose (%)†	Sex	Effective no. of rats‡	Total nitrite or nitrate consumed (g/rat)	No. of rats with tumours (%)	Survival time (wk) Mean ± SD	Range
			Sodium nitrite			
0 (control)	M	46	0	46 (100)	108 ± 13·6	72–120
	F	49	0	45 (92)	113 ± 10·6	82–120
0·125	M	49	19	49 (100)	109 ± 13·9	72–120
	F	48	15	41 (85)	114 ± 9·8	84–120
0·25	M	50	34	50 (100)	113 ± 13·2	56–120
	F	48	25	35 (73)*	112 ± 12·7	58–120
			Sodium nitrate			
0 (control)	M	50	0	47 (94)	108 ± 16·8	44–123
	F	50	0	46 (92)	105 ± 17·4	63–123
2·5	M	50	277	50 (100)	116 ± 9·3	90–123
	F	50	190	43 (86)	113 ± 15·6	68–123
5·0	M	50	575	48 (96)	109 ± 20·1	36–123
	F	49	394	39 (80)	109 ± 20·7	39–123

†The dose is expressed as the percentage in the drinking-water (sodium nitrite) or the percentage in the diet (sodium nitrate).
‡Initially each group comprised 50 animals.
The value marked with an asterisk differs significantly ($P < 0·05$; chi-square test) from the corresponding control value.

cantly decreased ($P < 0.05$) compared with that in the control group in the nitrite study, but there were no significant differences in the nitrate study. There was no significant difference in mean survival times between control and experimental groups in either study.

Tables 3 and 4 show the mean incidences and induction times of tumours of various organs and/or tissues. As shown in the tables, in all groups of males in both studies, tumours of the testis were the most frequent, followed by those of the mammary gland, adrenal gland and liver. Tumours of the mammary gland, pituitary gland, uterus, and adrenal gland were those detected most frequently in the female rats of both studies. Tumours were also detected in other organs and/or tissues of each group of both studies, although the incidence was relatively low. None of the treatment groups showed a significant increase in the incidence of any specific tumour compared with the corresponding control group. There was also no significant difference between experimental and control groups in the mean induction time of any tumour.

The only interesting difference between treated animals and controls is in the incidence of tumours of the haematopoietic organs in both studies. The incidence of these tumours was relatively high in the control groups of both sexes in both studies. The incidence was, however, significantly decreased in experimental groups, especially in the nitrate study.

Table 5 shows the age-related incidence of tumours of the testis, uterus, haematopoietic organs, pituitary gland, adrenal gland, liver and thyroid gland. These tumours increased rapidly after about 2 yr in each group of both studies and there was no particular tumour which seemed to appear earlier in each experimental group.

The tumours found in the two studies were histologically similar. All the testicular tumours that were detected were interstitial cell tumours. The mammary tumours differed histologically in male and female rats. In the male rats the epithelial element of the fibroadenoma was strongly atrophic and only the mesenchymal element proliferated as if it was a fibroma whereas typical fibroadenomas were the most frequent mammary tumours in the females. Most of the pituitary tumours were chromophobic adenomas. The tumours observed in the haematopoietic organs were mononuclear cell leukaemias, except for one other tumour in a male given the lower dose of nitrite. In cases of mononuclear cell leukaemia, clinical signs such as severe emaciation, anaemia, jaundice and abdominal distension were observed and at autopsy splenomegaly or hepatosplenomegaly was marked. Enlargement of lymph nodes was not so marked, although in a few cases systemic enlargement of lymph nodes was detected. Atypical mononuclear cells like monocytes or lymphocytes were detected in peripheral blood and in many cases erythrophagia was observed. Histochemical tests for peroxidase were negative. Invasion of leukaemic cells was observed in the spleen and liver with few exceptions and in some cases tumour-cell invasion was also observed in lymph nodes, lungs, kidneys, adrenal glands and bone marrow. Most adrenal tumours were small phaeochromocytomas, although there were a few cases of phaeochromocytomas combined with ganglioneuromas, and large malignant phaeochromocytomas with metastases in remote organs; cortical tumours were very rare. Most liver tumours were benign adenomas, although a few hepatocellular carcinomas were also observed. In the thyroid gland, C-cell adenomas were most common, although other types of thyroid tumours such as papillary adenomas and C-cell carcinomas were also observed. Endometrial angiomatous polyps, adenomas and adenocarcinomas were frequent in the uterus. The histology of the other tumours detected was similar to that of the spontaneous tumours reported by other investigators (Moloney, Boschetti & King, 1970; Sass, Rabstein, Madison *et al.* 1975).

There was no significant difference in the incidence of malignant tumours between experimental and control groups of both studies.

Table 6 shows the results of analysis for *N*-nitrosamines of the basic diet, diet containing nitrate, drinking-water containing nitrite, and the stomach contents of treated rats. More NDMA was detected in the nitrate-containing diet than in the basic diet. The maximum value, 37 ppb NDMA, was detected in the stomach contents of rats given sodium nitrite in their drinking-water and up to 11 ppb was found in the stomach contents of rats given nitrate in the diet, although no nitrosamine was detected in the stomach contents of control rats. No other *N*-nitroso compounds were detected.

DISCUSSION

Druckrey, Steinhoff, Buethner *et al.* (1963) reported that sodium nitrite was not carcinogenic in rats when it was given continuously in the drinking-water for their life-span. It has since been suggested that sodium nitrite is mutagenic in various mutagenicity tests (Odashima, 1980). Inai, Aoki & Tokuoka (1979) recently reported a carcinogenicity test in ICR mice in which sodium nitrite was given at levels of 0·5, 0·25 and 0·125% in the drinking-water for 109 wk and carcinogenicity was not detected. On the other hand, Newberne (1979) reported that sodium nitrite promoted lymphomas in experimental groups when Sprague–Dawley rats were given 250–2000 ppm of sodium nitrite in the diet or drinking-water but Newberne's data have since been invalidated (Dickson, 1980). In addition, Inui also reported transplacental action of nitrite on hamster embryo cells (Inui, Nishi, Taketomi & Mori, 1979). Most recently, Mirvish, Bulay, Runge & Patil (1980) reported that in MCR Wistar rats receiving sodium nitrite in drinking-water (3 g/litre) 8 of 45 rats (18%) had papillomas of the forestomach and the incidence was significantly greater than that in the control group (2%). However, there have been no significant reports on the carcinogenicity of sodium nitrate, although Sugiyama, Tanaka & Mori (1979) reported recently that sodium nitrate had been found to be non-carcinogenic in ICR mice.

In our studies many different types of tumours were observed in all groups including the controls. The distribution and histology of those tumours were similar to those of the spontaneous tumours mentioned by other investigators (Moloney *et al.* 1970; Sass *et al.* 1975). In our studies the incidence of spontaneous

Table 3. *Organ distribution and first time of detection of tumours in F-344 rats treated with sodium nitrite*

Treatment group ... Affected organ or tissue	Control: No. of tumour-bearing animals (%)	Control: Time to first tumour (wk)	0·125%: No. of tumour-bearing animals (%)	0·125%: Time to first tumour (wk)	0·25%: No. of tumour-bearing animals (%)	0·25%: Time to first tumour (wk)
		Males				
Testis	46 (100)	72	46 (94)	72	48 (96)	80
Mammary gland	9 (20)	91	15 (31)	91	13 (26)	108
Pituitary gland	5 (11)	72	5 (10)	88	3 (6)	80
Haematopoietic organs	16 (35)	72	5 (10)*	94	5 (10)*	105
Adrenal gland	9 (20)	98	11 (22)	94	5 (10)	118
Liver	4 (9)	115	5 (10)	98	7 (14)	119
Thyroid gland	4 (9)	102	4 (8)	97	0	
Peritoneum	1 (2)		4 (8)	87	2 (4)	101
Nervous system	1 (2)		1 (2)	120	1 (2)	56
Subcutaneous tissue	9 (20)	80	4 (8)	72	3 (6)	116
Lung	2 (4)	111	3 (6)	111	2 (4)	117
Preputial gland	3 (7)	84	3 (6)	85	7 (14)	84
Pancreas	3 (7)	91	5 (10)	111	4 (8)	108
Mediastinum	1 (2)	113	0		0	
Tongue	1 (2)	98	0		0	
Spleen	1 (2)	120	3 (6)	84	0	
Forestomach	1 (2)	103	0		0	
Skin	1 (2)	120	0		1 (2)	117
Heart	0		1 (2)	120	0	
Ear duct	0		0		1 (2)	101
Small intestine	0		0		1 (2)	120
Large intestine, rectum	0		0		2 (4)	88
Urinary bladder	0		1 (2)	120	0	
Kidney	0		0		2 (4)	119
Prostate	0		1 (2)	97	0	
		Females†				
Mammary gland	24 (49)	89	22 (46)	110	14 (29)*	80
Pituitary gland	17 (35)	86	11 (23)	101	14 (29)	80
Haematopoietic organs	13 (27)	82	7 (15)	95	4 (8)*	102
Adrenal gland	2 (4)	112	3 (6)	112	5 (10)	101
Liver	1 (2)	120	0		0	
Thyroid gland	4 (8)	100	3 (6)	120	3 (6)	101
Uterus	9 (18)	100	10 (21)	85	4 (8)	98
Nervous system	1 (2)	115	0		1 (2)	58
Subcutaneous tissue	0		1 (2)	120	2 (4)	118
Lung	3 (6)	116	3 (6)	120	1 (2)	120
Clitoral gland	0		1 (2)	114	4 (8)	101
Pancreas	2 (4)	89	1 (2)	120	0	
Forestomach	0		1 (2)	85	0	
Small intestine	0		1 (2)	109	1 (2)	120
Urinary bladder	0		1 (2)	112	0	

†Among females there were no tumours of the peritoneum, mediastinum, tongue, spleen, skin, heart, large intestine, rectum or kidney.
Values marked with asterisks differ significantly ($P < 0·05$; chi-square test) from the corresponding control values.

Table 4. *Organ distribution and first time of detection of tumours in F-344 rats treated with sodium nitrate*

Treatment group . . .	Control		2·5%		5·0%	
Affected organ or tissue	No. of tumour-bearing animals (%)	Time to first tumour (wk)	No. of tumour-bearing animals (%)	Time to first tumour (wk)	No. of tumour-bearing animals (%)	Time to first tumour (wk)
		Males				
Testis	44 (88)	78	49 (98)	90	39 (78)	98
Mammary gland	17 (34)	83	9 (18)	111	11 (22)	102
Pituitary gland	3 (6)	106	3 (6)	102	4 (8)	87
Haematopoietic organs	18 (36)	78	2 (4)*	90	1 (2)*	
Adrenal gland	9 (18)	101	14 (28)	101	12 (24)	98
Liver	6 (12)	106	7 (14)	116	4 (8)	101
Thyroid	1 (2)	78	6 (12)	108	6 (12)	98
Peritoneum	2 (4)	109	3 (6)	90	2 (4)	107
Nervous system	1 (2)	120	3 (6)	116	2 (4)	36
Subcutaneous tissue	2 (4)	44	5 (10)	92	3 (6)	100
Lung	2 (4)	120	2 (4)	116	3 (6)	78
Preputial gland	5 (10)	94	2 (4)	123	2 (4)	100
Pancreas	2 (4)	106	5 (10)	122	6 (13)	88
Tongue	1 (2)	113	0		0	
Spleen	0		3 (6)	113	0	
Skin	0		1 (2)	123	0	
Heart	0		1 (2)	123	0	
Ear duct	1 (2)	90	0		0	
Small intestine	0		0		1 (2)	100
Large intestine, rectum	0		0		2 (4)	101
Salivary gland	0		1 (2%)	108	0	
		Females†				
Mammary gland	20 (40)	75	20 (40)	104	13 (27)	68
Pituitary gland	17 (34)	89	18 (36)	74	11 (22)	85
Haematopoietic organs	14 (28)	74	0*		1 (2)*	119
Adrenal gland	4 (8)	70	6 (12)	79	4 (8)	115
Liver	2 (4)	63	0		0	
Thyroid gland	2 (4)	91	0		1 (2)	123
Uterus	4 (8)	70	10 (20)	74	6 (12)	98
Nervous system	0		2 (4)	87	1 (2)	45
Subcutaneous tissue	2 (4)	103	4 (8)	89	1 (2)	95
Lung	0		2 (4)	123	2 (4)	123
Clitoral gland	3 (6)	123	3 (6)	68	6 (12)	68
Pancreas	0		1 (2)	123	1 (2)	123
Spleen	0		0		1 (2)	115
Skin	0		1 (2)	121	0	
Ear duct	1 (2)	93	1 (2)	93	0	
Small intestine	0		0		1 (2)	109
Ovary	1 (2)	95	1 (2)	123	0	
Salivary gland	1 (2)	123	0		0	

†Among females there were no tumours of the peritoneum, tongue, heart, large intestine or rectum.
Values marked with asterisks differ significantly ($P < 0{\cdot}01$; chi-square test) from the corresponding control values.

Table 5. *Age-related incidence of tumours in F-344 rats treated with sodium nitrite or sodium nitrate*

		No. of rats with tumours of the																				
			Testis or uterus			Mammary gland			Haemato-poietic organs			Pituitary gland			Adrenal gland			Liver			Thyroid	
Sex	Age group (months)	Treatment group ...	C	L	H	C	L	H	C	L	H	C	L	H	C	L	H	C	L	H	C	L	H
		Sodium nitrite study																					
Male	13–15																						
	16–18		2	1					1			1											
	19–21		3	4	3								1	1									
	22–24		11	7	3	3	1		4	1		1	1		4	1			1		1	1	
	25–27		13	8	10	3	2	5	6	3	4	1	2	1	1	2		1	1			1	
	28–		17	26	32	3	22	9	5	1	1	2	1	1	4	8	5	3	3	7	3	2	
Female	13–15																						
	16–18																						
	19–21		1	1		1		1	2	1		2		2									
	22–24		1	1	2		1	2	1	1	1	2	1	1			2				1		1
	25–27		5	2		10	3	1	6	3	3	3	4	4	1	1	1				1		
	28–		2	6	2	13	18	10	4	2		10	6	7	1	2	2	1			2	3	2
		Sodium nitrate study																					
Male	13–15																						
	16–18		1						1												1		
	19–21		1	1		2			1	1				1									
	22–24		10	5	7	4		2	4				1	1	2		3			1			2
	25–27		13	12	5	3	3	1	9	1		1			3	6	2	1	1			2	
	28–		19	31	27	8	6	8	3		1	2	2	2	4	8	7	5	6	3		4	4
Female	13–15																	1					
	16–18		2	1		1		1	1				1		2								
	19–21					1		1	1			1	3	1		1		1					
	22–24		2		1	5	1	3	6			7	2	2							2		
	25–27			1		5	1	2	3			3	2	2	1								
	28–			8	5	8	18	6	3		1	6	10	6	1	5	4						1

C = Control group L = Low-dose group H = High-dose group

Table 6. *Determination of* N-*nitroso compounds in the diets, drinking-water and stomach contents of nitrite- and nitrate-treated and control rats*

Material analysed	NDMA (ppb)	
Basic diet (CRF-1)	7·5	ND
Diet containing 2·5% nitrate	14·4	49·2
Diet containing 5% nitrate	12·9	27·5
Water containing 0·125% nitrite	ND	ND
Water containing 0·25% nitrite	ND	ND
Stomach contents of control males	ND	ND
Stomach contents of control females	ND	ND
Stomach contents of 2·5% nitrate males	Trace	
Stomach contents of 2·5% nitrate females	10·7	
Stomach contents of 5% nitrate males	10·1	6·8
Stomach contents of 5% nitrate females	10·0	10·8
Stomach contents of 0·125% nitrite males	Trace	
Stomach contents of 0·125% nitrite females	9·0	
Stomach contents of 0·25% nitrite males	36·8	18·0
Stomach contents of 0·25% nitrite females	13·5	15·2

ND = Not detected NDMA = *N*-Nitrosodimethylamine
Nitrosamines other than NDMA were not detected.

tumours in the controls was very high. Comparing the total incidence of tumours in treated and control groups is therefore of little value. None of the tumour types had a significantly higher incidence among treated animals than among controls. The age-related tumour incidences also demonstrated a lack of effect of the treatments.

The most interesting result in the intergroup difference in the incidence of leukaemias. The incidence of mononuclear cell leukaemias was relatively high in all the control groups. F-344 rats have a high incidence of spontaneous mononuclear cell leukaemias. The spontaneous incidence has been reported as 31% in males and 21% in female rats (Sass *et al.* 1975) and as about 25% (Moloney, 1970). The incidence of the leukaemias in the controls in our studies was about the same or slightly above these previous reports but the incidence in experimental groups was much lower. The reason for this reduction in the incidence of spontaneous leukaemias is not clear but in treated rats there was slight atrophy of the haematopoietic organs such as the spleen and lymph nodes. This finding may be important in the reduction of leukaemias.

Our results were widely different from those of Newberne (1979) but as previously mentioned Newberne's data have been invalidated. Mirvish *et al.* (1980) reported that sodium nitrite induced papillomas of the forestomach in Wistar rats but in our sodium nitrite study only one female rat in the 0·125% group developed forestomach papillomas.

Sodium nitrite is a precursor of *N*-nitroso compounds and in combination with secondary amines can form nitrosamines in food or in the body. Sodium nitrate is also a precursor of *N*-nitroso compounds since it can be reduced to the nitrite either in the body or in food (Heisler *et al.* 1974; Ishiwata *et al.* 1975).

Aoyagi, Matsukura, Uchida *et al.* (1980) recently reported induction of liver tumours in Wistar rats by sodium nitrite given in pelleted diet. Volatile *N*-nitroso compounds, especially NDMA were detected at ppm levels in the pelleted diet used and they discussed the possibility that *N*-nitroso compounds formed in the treated diets were the main cause of liver tumours.

In our studies, there was more NDMA in the diet containing sodium nitrate than in the basic diet, and NDMA was detected in the stomach contents of rats given sodium nitrite or nitrate, although no nitrosamine was detected in the stomach contents of control animals. But the amount of NDMA detected in our studies was very low compared with the minimum carcinogenic dose reported by Terracini, Magee & Barnes (1967). It is well known that the main target organs of NDMA are the liver and the kidney in rats. In our studies the incidence of liver tumours in experimental groups was no higher than that among the controls and there was also no treatment-related effect on the incidence of kidney tumours. These results suggest that the tumours including liver tumours observed in our studies were not due to NDMA.

It is concluded that sodium nitrite and sodium nitrate did not have carcinogenic activity in F-344 rats when they were administered continuously in the drinking water or diet for 2 yr. It is, however, very important to reduce the level of these chemicals in foods, because it is known that they are precursors of the carcinogenic *N*-nitroso compounds.

Acknowledgement—We thank Dr A. Tanimura, Chief, Division of Food Additives, National Institute of Hygienic Sciences, for testing the stability of sodium nitrate in the diet and for the analysis of nitrosamines in the diets, drinking-water and stomach contents.

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Mutation Research, 242 (1990) 181–186
Elsevier

MUTGEN 01595

Formation of mutagens during the frying of Hawaiian fish: correlation with creatine and creatinine content

Nancy L. Marsh [a], Wayne T. Iwaoka [b] and Howard F. Mower [a,c]

[a] *Department of Biochemistry and Biophysics, J.A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96822,* [b] *Department of Food Science and Human Nutrition, College of Tropical Agriculture and Human Resources, University of Hawaii, Honolulu, Hawaii 96822 and* [c] *Cancer Research Center of Hawaii, University of Hawaii, Honolulu, Hawaii 96813 (U.S.A.)*

(Received 24 July 1989)
(Revision received 7 May 1990)
(Accepted 14 May 1990)

Keywords: Fish, frying, formation of mutagens; Creatine; Creatinine

Summary

Compounds mutagenic toward *Salmonella typhimurium* strain TA98 in the presence of rat-liver homogenates (S9) were formed when fish flesh was fried at 199°C. Three species of Hawaiian fish commonly consumed in Hawaii (skipjack tuna, *Katsuwonus pelamis*; yellowfin tuna, *Neothunnus macropterus*; and milkfish, *Chanos chanos*) were cooked in an electric skillet, along with samples of sole (*Microstomus pacificus*). Organic extracts of the fish were tested in the Ames Salmonella mutagenic assay using tester strain TA98 and S9. Basic organic extracts of fried, but not raw, samples exhibited significant mutagenicity. The levels of mutagenicity were also higher among the red flesh Hawaiian fish ('ahi, aku and awa) than with the white flesh sole. Creatine and creatinine contents were highest in the Hawaiian fish and lower in the sole. Creatine levels in the fish were 50–100 times greater than the creatinine content and varied from a high of 645 mg/100 g wet weight of fish for yellowfin tuna to a low value of 251 mg/100 g for sole. Mutagen levels are only approximately related to creatine/creatinine levels suggesting that other components contained in these fish may be as important as the guanidines in determining the levels of mutagen in the cooked fish.

Through previous studies, it has been shown that the cooking procedure has a strong influence on the mutagenicity of a food item. For example, beefsteak, hamburger and fish that had been grilled over a gas flame were found to contain high levels of mutagens on their charred surface (Nagao et al., 1977; Commoner et al., 1978; Sugimura, 1979). Beefsteaks, sausages and pork chops grilled over charcoal also contained potent mutagenic polycyclic aromatic hydrocarbons (Lijinski and Shubik, 1964, 1965; Fabian, 1968; Fritz, 1973). In addition, heated beef and beef extract were also found to be mutagenic (Commoner et al., 1978). A survey of the major sources of cooked protein foods in the American diet by Felton et al. (1982a) showed that most meats, when cooked to a well-

Correspondence: Dr. Nancy L. Marsh, Department of Biochemistry and Biophysics, J.A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96822 (U.S.A.).

0165-1218/90/$03.50

done but non-charred state, contain mutagens active in the Salmonella bioassay. The first signs of charring on samples of chicken, eggs, beef, and pork were associated with high levels of activity. For most of these samples, mutagenicity tended to increase with increasing charring. In another survey by Felton et al. (1982b), seafood samples (red snapper, salmon, trout, halibut and rock cod) gave a variety of results, but all gave more than 1000 revertants/100 g wet weight equivalents using TA1538 when pan-fried or griddle fried for about 6 min/side. These results are similar to ours since more than 1000 revertants/100 g wet weight equivalents were produced using TA98.

In general, cooking methods that use temperatures near 100 °C result in low or nondetectable activity. The processes of stewing, boiling and poaching, which cook near 100 °C, result in low mutagenic activity regardless of the type of food (Felton et al., 1982a). It has been reported that microwave cooking, also a low-temperature process, produces no mutagenic activity (Dolara et al., 1979). Cooking methods such as oven roasting and baking, which heat food by indirect convection, appeared to produce low to intermediate levels of mutagenic activity in most foods. Frying and broiling, cooking procedures that heat foods by conductive and radiative processes, tended to be associated with the highest levels of mutagenicity. Thus, the rates of mutagen production in the tested foods become appreciable at temperatures greater than 100 °C, and are greatly increased by cooking methods that use direct high-temperature processes (Felton et al., 1982a).

As a result of these findings, food typical to the Hawaiians' diet has been researched for possible mutagens. In a previous study, common Hawaiian fish that are eaten dried and salted were examined and found to contain mutagenic substances (Mower and Ichinotsubo, 1982). In our study on mutagen formation during the frying of fish, three fish commonly eaten in Hawaii and elsewhere in the Pacific were fried and their levels of mutagenicity compared to their raw state. Two of the fish ('ahi or yellowfin tuna and aku or skipjack tuna) together account for more than half of the total pounds of commercial fish caught in Hawaii in 1986 (Department of Business and Economic Development, 1987).

Materials and methods

The fish fillets, skipjack or striped tuna, *Katsuwonus pelamis* (local name aku), yellowfin tuna, *Neothunnus macropterus* (local name 'ahi), and milkfish, *Chanos chanos* (local name awa), were obtained from a local market in fresh condition. The sole, *Microstomus pacificus*, was also obtained from a local market but frozen. All chemicals used were ACS grade or better. The dichloromethane was Baker 'Analyzed.'

Analysis of creatine and creatinine

Creatine and creatinine were analyzed in fresh uncooked fish using the Sigma Chemical Co. diagnostic kit No. 555. The analytical procedure of this kit was changed to allow the measurement of creatine as well as creatinine. This was done by using the procedure of Lambert (1945). A 2–3 g sample was cut from the fish and homogenized in 5 vol. of creatinine color reagent [0.6% picric acid and sodium borate at pH 8.25 (reagent No. 555-1)]. The homogenate was then divided into two approximately equal portions. One portion was autoclaved for 80 min to convert the creatine of the sample to creatinine. After cooling of the autoclaved sample, both samples were then centrifuged for 10 min at $7000 \times g$ and the supernatants were analyzed according to the Sigma procedure.

Cooking procedure

Samples of each of the four fish were fried in an electric skillet, lightly greased with vegetable oil. The 'ahi, aku and awa were fried in fillets of 0.5 inch (average weight of 67 g) for 6 min on each side, 4–5 fillets at a time, at a temperature of 199 °C. Fish fillets are commonly prepared by frying at temperatures of about 200 °C (Iwaoka and Krone, 1981). The sole was also fried in a similar manner but for 4 min per side, a time which has been shown to yield the maximum number of mutagens for sole (Iwaoka and Krone, 1981). These conditions produced a final cooked fish that was considered adequately cooked but not 'overdone' or charred. The temperature during frying was recorded at the surface of the fillet in contact with the skillet and measured with a Temperature Indicator Potentiometer (Leeds and

Northrup Co., Philadelphia, PA) using an iron constantan thermocouple.

Isolation and concentration of mutagens

Samples of raw fish of each species and their respective cooked samples (including the added oil) were homogenized with 2–3 times the volume equivalents of methyl alcohol in a Waring blender, then filtered through glass wool in a Buchner funnel with slight suction. The homogenization was repeated and the filtrate was then placed in a rotary evaporator and the solvent removed at reduced water pump pressure at 35–40 °C. Distilled H_2O, 150 ml, was then added to dissolve the residue and the solution was acidified to pH of 2.5 with the addition of HCl. After being extracted 3 times with dichloromethane, the acidic organic extract was dried over sodium sulfate and the dichloromethane removed by rotary evaporation. The aqueous extract was adjusted to a pH of 10.5 by the addition of NaOH and extracted another 3 times with dichloromethane. The basic organic extract was also dried over sodium sulfate and the dichloromethane removed by rotary evaporation. The residues were taken up in volumes of 15 ml of dichloromethane. This extraction procedure has been studied extensively and found to concentrate mutagens from cooked fish without producing artifactual mutagenicity (Iwaoka and Krone, 1981).

Mutagenesis assay

Aliquots of the organic extracts were placed in 13 × 100 mm sterile culture tubes, the dichloromethane removed under a stream of dry nitrogen and 250 μl of dimethyl sulfoxide added. These residues were then tested for mutagenicity according to the procedure described in Ames et al. (1975) using *Salmonella typhimurium* strain TA98 with the addition of 100 μl S9 mix containing 10% Aroclor-induced rat-liver S9 preparation. All experiments were repeated at least once. Two or more plates for all dose levels were included in each run. Plates were examined with a dissecting microscope for any indication of background lawn toxicity. Spontaneous revertants, in the absence of S9, were 38 ± 5 for extracts of fried awa and 'ahi and 30 ± 5 for extracts of fried aku and sole. Positive controls (−S9) were 2,4,7-trinitro-9-fluorenone (2,4,7-TNFone) (−S9) and 2-aminofluorene (2-AF) (+S9). The number of revertant colonies routinely produced with these controls was similar to published values (Maron and Ames, 1983). Cultures of TA98 were routinely examined for ampicillin resistance and the deep rough (*rfa*) character (Maron and Ames, 1983). Results were recorded as mutagenic activity ratio (MAR). This was calculated by dividing the number of revertants on the test plate by the number of colonies on the solvent control plate. Significant mutagenicity was present in an extract if the mutagenic activity ratio (MAR) > 2.0. The MAR was often referred to the gram equivalent (gE) of the extract applied to the test plate. The gE is the weight of cooked fish that was processed to give the extract that was applied to the test plate.

Results

The results of the creatine and creatinine analysis of each fish are shown in Table 1. Creatine levels exceed those of creatinine by factors of 50–60 for each of the fish.

Mutagenic activity was observed in the basic organic extracts from fried samples of all fish species tested (Fig. 1). Metabolic activation with rat-liver homogenates (S9) was required to produce the mutagenicity. Basic extracts from the uncooked samples, as well as the acid extracts from all samples, exhibited little or no mutagenicity, even with the inclusion of microsomal enzymes.

The frying of 'ahi, aku and awa produced higher levels of mutagenicity than that of the sole. In an earlier study by Iwaoka and Krone (1981), sole that was fried for the same time (4 min per side)

TABLE 1

CREATINE, CREATININE AND MOISTURE CONTENT OF FISH

Fish	%water	Creatine [a]	Creatinine [a]
'Ahi	74.1	645.8	12.9
Aku	72.6	272.8	4.4
Awa	77.8	411.2	4.5
Sole	82.9	251.0	2.5

[a] mg/100 g wet weight fish.

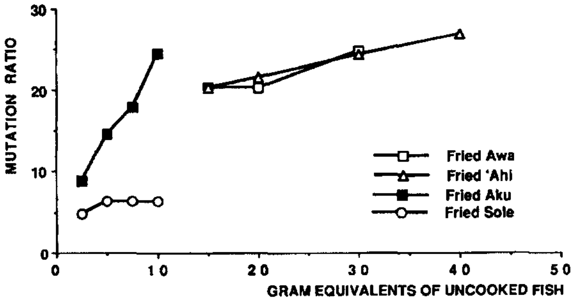

Fig. 1. Mutagens in fried fish.

in a similar manner but at 190 °C produced a MAR of 7.8 for basic extract from 20 gE (gram equivalents) uncooked fish (extract obtained from the final DMSO solution of 20 g of fish) using strain TA98 with S9. In our study (Table 2), a MAR of 12 was obtained under similar conditions but at 9 °C higher. Although a small increase in temperature produced a significant increase in MAR for the sole, the MAR values are much greater in the red flesh Hawaiian fish at a slightly higher temperature and cooking time when compared to several white or red flesh fish from a previous study (Iwaoka and Krone, 1981). For 20 gE uncooked fish, the MAR of the awa, 'ahi and aku are 20, 22 and 56, respectively. These values are much higher than those obtained by Iwaoka and Krone for 20 gE sole, salmon, turbot, and snapper (Table 2).

TABLE 2

MUTAGENIC ACTIVITY OF FISH CAUGHT IN TROPICAL WATERS vs. FISH CAUGHT IN COLDER WATERS USING 20 gE (GRAM EQUIVALENTS) UNCOOKED FISH

Fish	Time fried (min/side)	Temperature	MAR
Awa [a]	6	199 °C	20
'Ahi [a]	6	199 °C	21
Aku [a]	6	199 °C	56
Sole [a]	4	199 °C	12
Sole [b]	4	190 °C	7.8
Salmon [b]	4	190 °C	7.1
Turbot [b]	4	190 °C	2.8
Snapper [b]	4	190 °C	6.0

[a] Our study.
[b] Iwaoka and Krone (1981)

TABLE 3

MUTAGENICITY ACTIVITY OF FISH CAUGHT IN TROPICAL WATERS vs. FISH CAUGHT IN COLDER WATERS USING 100 gE (GRAM EQUIVALENTS) UNCOOKED FISH

Fish	Time fried (min)/side	Temperature	MAR
Awa [a]	6	199 °C	134
'Ahi [a]	6	199 °C	135
Aku [a]	6	199 °C	282
Sole [a]	4	199 °C	116
Rock cod [b]	6	280 °C	60.4
Trout [b]	6.5	280 °C	140.9
Salmon [b]	6	280 °C	127.3
Red snapper [b]	5.5	280 °C	113.6

[a] Our study.
[b] Felton et al. (1982a).

In a study by Felton et al. (1982a) using 100 gE uncooked fish and strain TA1538, the MAR values for rock cod, trout, salmon, turbot and red snapper that had been fried at a considerably higher temperature are not very much higher than those of the awa, 'ahi and aku (Table 3). In general, the MAR values obtained using TA98 and TA1538 are very similar (Iwaoka and Krone, 1981), but one would expect a significant increase in MAR with an increase in temperature of 81 °C. Instead, trout fried for 6.5 min/side at 280 °C yielded a MAR of 140.9, which is only slightly higher than the awa and 'ahi fried for 6 min/side at 199 °C with a MAR of 134 and 135, respectively. The MAR values for the other white or red flesh fish fried at 280 °C are all lower than those of the red flesh Hawaiian fish fried at 199 °C (Table 3). In summary, 2–8 times more mutagens were formed in red flesh fish at slightly higher cooking temperatures and cooking times than in the fish studied by Iwaoka and Krone, and about the same number of mutagens were formed at cooking temperatures 80 °C less than in the fish studied by Felton et al. (1982a).

Discussion

Fish consumption has been steadily rising in the U.S.A. The recent information about the advantages of the ω-3 polyunsaturated fatty acids from certain ocean fish in reducing the risk of

heart disease undoubtedly has contributed to this increasing trend. However, eating large amounts of fish may not be without some disadvantages, particularly if certain cooking processes are used. In our study, the effects of frying as a procedure of cooking fish resulted in the formation of compounds with significant mutagenicity, as had been formed previously in the other studies on the effects of cooking on high-protein foods. In general, during the frying of the fish, there was a considerable amount of water loss from the tissues. Foods having a high protein content with a low water content were shown to give high levels of mutagenicity (Uyeta et al., 1979). In the study by Felton et al. (1982a), there was an indication that fish containing more pink or red color in the flesh were more susceptible to the formation of mutagens than white flesh fish. This study seems to support that finding.

The fried red flesh of the 'ahi, aku and awa appears to have a greater level of mutagenicity than the lighter colored flesh of sole, possibly indicating the presence of a greater number of precursor compounds in the Hawaiian fish. It has been reported by a number of investigators (see C. de Meester, 1989 for a summary of these observations) that creatine and creatinine can react with a variety of simple sugars and amino acids to form IQ, MeIQ, 4,8-DiMeIQ and PhIP. We therefore measured the amounts of these guanido derivatives in the fish we are studying in this report. The results, shown in Table 1, indicate as expected that sole contains lower amounts of creatine and creatinine than do the Hawaiian fish. Among the red fleshed fish, 'ahi contains the highest amount of the guanido derivatives. The levels that we report here are ~ 78% higher than those reported by T. Suzuki et al. (1989). These high levels in 'ahi are, however, not translated into proportionally higher levels of mutagens compared to aku and awa which contain much lower amounts of creatine and creatinine but form as high a level of mutagens on cooking as does 'ahi. This discrepancy may be due to limiting amounts of coreactants, such as sugars or appropriate amino acids in the 'ahi.

In the U.S.A., over 80% of tuna catches are processed by canning and 1.4 billion pounds of canned fishery products are produced annually (Anon., 1982). However, oil packed tuna has a MAR of only 3.8 for 80 gE raw fish and water packed tuna contains no detectable or very low levels of mutagens (Krone and Iwaoka, 1987). Perhaps it is because of the low temperature used (102°C) and the procedure of steaming, as opposed to frying, in the precooking process (Wheaton and Lawson, 1985) that the extent of mutagen formation is low in canned tuna.

The mutagens produced during the cooking, but not charring, of high-protein foods such as fish, meat and poultry were found to be due to the presence of imidazo-quinoline/quinoxaline and imidazo-pyridine type compounds. Examples of these types of mutagens which have been found include IQ (2-amino-3-methyl-imidazo[4,5-*f*]-quinoline), MeIQ (2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline), MeIQx (2-amino-3,8-dimethyl-imidazo[4,5-*f*]quinoxaline), and DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline) (Kasai et al., 1980a,b, 1981; Becher et al., 1988). The specific mutagenic activities of IQ, MeIQ and MeIQx are very high towards *S. typhimurium* TA98 with S9, showing 433 000, 661 000 and 145 000 revertants/μg, respectively (Sugimura, 1986). IQ has been shown to be a strong inducer of unscheduled DNA synthesis in liver cells and is carcinogenic in mice and rats (Barnes et al., 1985; Ohgaki et al., 1984; Takayama et al., 1984). MeIQ added to the diets of mice induced tumors in the forestomach and livers of these animals (Ohgaki et al., 1985). Recently, substituted imidazopyridines such as TMIP (2-amino-*N*,*N*,*N*-trimethylimidazopyridine), DMIP (2-amino-*N*,*N*-dimethylimidazopyridine), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine have been found to be mutagenic (Becher et al., 1988).

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Clinical and Experimental Pharmacology and Physiology (2002) **29**, 782–783

SHORT COMMUNICATION

CONTRACTILE PROPERTIES OF THE DIAPHRAGM IN CREATINE-FED RATS

M McGuire,* A Bradford and M MacDermott

Department of Physiology, Royal College of Surgeons in Ireland, Dublin, Ireland

SUMMARY

1. Creatine feeding increases the oxidative capacity of type 1 skeletal muscle fibres and, in soleus muscles, consisting mainly of type 1 fibres, increases fatigue resistance. The diaphragm contains a relatively large content of type 1 fibres and respiratory muscle fatigue is a cause of respiratory failure. The aim of the present study was to determine whether creatine supplements increase fatigue resistance in the diaphragm.

2. Rats were given creatine monohydrate (2.55 g/L) in the drinking water. After 5–6 days, isometric contractile properties were measured in strips of costal diaphragm in Krebs' solution at 30•C. Measurements were also made in soleus muscle strips. Values for strips from creatine-fed rats were compared with those from control rats.

3. Creatine feeding did not increase fatigue resistance and had no effect on twitch or tetanic tension or twitch kinetics in the diaphragm. Creatine increased fatigue resistance in soleus muscles, as reported previously.

Key words: contraction, creatine, diaphragm.

INTRODUCTION

Creatine exists in skeletal muscle as free creatine (Cr) and phosphocreatine (PCr). Phosphocreatine is a rapidly available energy source for skeletal muscle contraction. In humans and rats, dietary Cr supplements increased Cr and PCr concentrations in skeletal muscle[1–3] and improved exercise performance.[1,3,4] In addition, Cr feeding in rats increased citrate synthase (CS) activity in type 1 fibres,[3] indicating increased oxidative capacity, but had no effect on CS activity in type 2 fibres.[3] Consistent with this, Cr feeding increased fatigue resistance in soleus muscles[5] containing 84% type 1 fibres,[6] but had no effect on fatigue resistance in extensor digitorum longus (edl) muscles[5] containing 4% type 1 fibres.[6]

Forty-five per cent of fibres in the rat costal diaphragm are type 1 fibres,[7] so Cr feeding could increase CS activity and, consequently, fatigue resistance in this muscle. Therefore, in the present study we have examined the effects of dietary Cr supplements on the contractile properties of the diaphragm. Because respiratory muscle fatigue is one of the causes of respiratory failure,[8] the possibility that Cr may improve fatigue resistance in the diaphragm was worth investigating. In some experiments, fatigue resistance in soleus muscles was evaluated because this was shown previously to increase following Cr feeding.[5]

METHODS

All procedures were performed in accordance with national legislation under the Cruelty to Animals Act, 1876 and EU Directive 86/609/EC. Treatment of the rats and measurement of contractile properties were as described previously.[5] Briefly, male Wistar rats (365–395 g) were randomly divided into two groups of seven. One group was given creatine monohydrate (2.55 g/L) in the drinking water. After 5–6 days, rats were anaesthetized (pentobarbitone sodium; 60 mg/kg, i.p.) and the soleus and diaphragm muscles were removed rapidly and placed in oxygenated (95% O_2–5% CO_2) Krebs' solution of the following composition (in mmol/L): NaCl 120; $NaHCO_3$ 25; $NaH_2PO_4.2H_2O$ 1.2; $MgSO_4.7H_2O$ 1.2; KCl 5.0; calcium gluconate 2.5; glucose 11.5. The diaphragm was divided along its central tendon and a small costal strip was dissected from one hemidiaphragm and suspended vertically in an organ bath containing oxygenated Krebs' solution maintained at 30°C and pH 7.4. The costal margin of the diaphragm strip was anchored to the base of the bath and the central tendon was attached to an isometric force transducer mounted on a micropositioner. Preparation of soleus muscle strips was as described previously.[5] In diaphragm muscle strips, isometric twitch tension, tetanic tension, contraction time, half-relaxation time, the tension–frequency relationship and fatigue were measured at resting length using field stimulation (supramaximal voltage, 1 msec duration) with platinum plate electrodes. Following an equilibration period of 30 min, a single twitch was elicited. The tension–frequency response was then determined using stimulation trains of 300 msec at frequencies of 20–100 Hz in increments of 20 Hz. Ten minutes after the tension–frequency determination, fatigue was induced using a fatigue protocol of 30 Hz trains every 2 s for 5 min. Fatigue, evaluated at 5 min of the fatigue protocol described above, was the only contractile measurement made in soleus muscle strips.

Data analysis

Specific tension was calculated in Newtons per strip cross-sectional area (N/cm^2). To calculate the latter, the muscle strip was blotted dry and weighed. The weight was then divided by the product of the optimal length and muscle density, assumed to be 1.056 mg/mm^3. For fatigue, values were

Correspondence: M MacDermott, Department of Physiology, Royal College of Surgeons in Ireland, 123 St Stephens Green, Dublin 2, Ireland. Email: mmacderm@rcsi.ie

*Present address: Sleep Disorders Program, Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.

Received 14 August 2001; revision 25 March 2002; accepted 10 April 2002.

Table 1 Effects of creatine feeding on the contractile properties of diaphragm muscle

	Twitch tension (N/cm^2)	Tetanic tension at 100 Hz (N/cm2)	Contraction time (s)	Half-relaxation time (s)	Fatigue (% initial tension)
Control ($n = 7$)	3.93 ±0.82	16.28 ±2.70	0.038 ±0.004	0.035 ±0.005	53.14 ±6.00
Cr fed ($n = 7$)	4.20 ±1.42	17.45 ±5.46	0.040 ±0.005	0.033 ±0.005	52.07 ±8.10

Values are the mean±SD, with n indicating the number of rats. Fatigue values are tension at 5 min of the fatigue protocol as a percentage of tension at 0 min.

Values for diaphragms from creatine (Cr)-fed rats were not significantly different from those of control rats.

normalized by expressing the tension generated by the stimulus trains at 1, 2, 3, 4 and 5 min as a percentage of that generated by the stimulus train at 0 min. Values for specific twitch and tetanic tensions, contraction times and half-relaxation times and fatigue were expressed as the mean±SD and used to compare statistically the control and creatine-fed groups using ANOVA. $P < 0.05$ was taken as significant.

RESULTS

Based on their water intake of 120 ± 9 mL/kg per day ($n = 7$), each Cr-fed rat consumed 0.307 ± 0.023 g Cr monohydrate/kg body-weight (equivalent to 0.270 g Cr/kg) per day.

In diaphragm muscles, Cr feeding had no effect on twitch or tetanic tension, twitch kinetics or fatigue resistance evaluated at 5 min of the fatigue protocol (Table 1). Fatigue resistance in the diaphragm evaluated at 1, 2, 3 and 4 min of the fatigue protocol was similarly unaffected by Cr feeding. Tension at these times, expressed as a percentage of tension at 0 min, was 101 ± 5, 86 ± 6, 68 ± 6 and 59 ± 7% ($n = 7$), respectively, compared with corresponding values in control diaphragms of 97 ± 4, 88 ± 3, 73 ± 4 and 61 ± 2% ($n = 7$). In soleus muscles, Cr feeding increased ($P < 0.05$) fatigue resistance. Tension at 5 min of the fatigue protocol, as a percentage of that at 0 min, was 74.5 ± 7% ($n = 4$) compared with a corresponding value of 58.7 ± 6% ($n = 3$) in control muscles.

DISCUSSION

Each rat consumed an average of 0.27 g Cr/kg daily. A similar daily intake for 4 days significantly increased Cr and PCr concentrations in rat skeletal muscles.[3] Therefore, it is reasonable to assume that the 5–6 days of Cr feeding in the present study would also have increased Cr and PCr in skeletal muscles. However, Cr feeding did not increase fatigue resistance in the diaphragm. It did increase fatigue resistance in soleus muscles, in agreement with previous results.[5] The effect in soleus muscles was attributed[5] to the fibre type-dependent increase in CS activity in soleus muscles of Cr-fed rats.[3] In the present study, the finding that Cr feeding did not increase fatigue resistance in the diaphragm, despite its large complement of type 1 fibres,[7] could suggest that Cr did not increase CS activity in this muscle. Because CS activity in the diaphragm[9] is almost twice that in soleus muscles,[3] a differential effect of Cr on CS activity in these two muscles could occur if the effect depended on the basal level of enzyme activity, as well as fibre type. Whatever the reason for the failure of Cr to increase fatigue resistance in the diaphragm, it is disappointing in that it excludes Cr as a potentially useful agent in attenuating respiratory muscle fatigue.

ACKNOWLEDGEMENTS

The authors thank the Royal College of Surgeons in Ireland for financial support and T Dowling and J Slattery for technical assistance.

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Critical Reviews in Biochemistry and Molecular Biology, 40:1–20, 2005

ISSN: 1040-9238 print / 1549-7798 online
DOI: 10.1080/10409230590918577

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Relating Structure to Mechanism in Creatine Kinase

Michael J. McLeish and George L. Kenyon
College of Pharmacy, University of Michigan, Ann Arbor, MI, USA

ABSTRACT Found in all vertebrates, creatine kinase catalyzes the reversible reaction of creatine and ATP forming phosphocreatine and ADP. Phosphocreatine may be viewed as a reservoir of "high-energy phosphate" which is able to supply ATP, the primary energy source in bioenergetics, on demand. Consequently, creatine kinase plays a significant role in energy homeostasis of cells with intermittently high energy requirements. The enzyme is of clinical importance and its levels are routinely used as an indicator of myocardial and skeletal muscle disorders and for the diagnosis of acute myocardial infarction. First identified in 1928, the enzyme has undergone intensive investigation for over 75 years. There are four major isozymes, two cytosolic and two mitochondrial, which form dimers and octamers, respectively. Depending on the pH, the enzyme operates by a random or an ordered bimolecular mechanism, with the equilibrium lying towards phosphocreatine production. Evidence suggests that conversion of creatine to phosphocreatine occurs via the in-line transfer of a phosphoryl group from ATP. A recent X-ray structure of creatine kinase bound to a transition state analog complex confirmed many of the predictions based on kinetic, spectroscopic, and mutagenesis studies. This review summarizes and correlates the more significant mechanistic and structural studies on creatine kinase.

KEYWORDS energy homeostasis, guanidino kinase, myocardial infarction, phosphagen kinase, phosphoryl group transfer, transition-state analogue complex, X-ray structure

INTRODUCTION

Creatine kinase (CK; adenosine-5′-triphosphate:creatine phosphotransferase; creatine phosphokinase; phosphocreatine phosphokinase; creatine *N*-phosphotransferase; EC 2.7.3.2) catalyzes the reversible transfer of a phosphoryl group from MgATP to creatine (Cr), producing phosphocreatine (PCr) and MgADP (Figure 1).

Phosphocreatine was initially identified in muscle tissue (Eggleton & Eggleton, 1928). At that time, it was thought to be the chemical source for the energy required for muscle contraction. However, not long after, the enzyme now known as creatine kinase was first identified (Lohman, 1934), and it was subsequently shown that ATP was formed by transfer of a phosphoryl

Editor: Michael M. Cox

Address correspondence to Michael J. McLeish, College of Pharmacy, University of Michigan, 428 Church St., Ann Arbor, MI. E-mail: mcleish@umich.edu

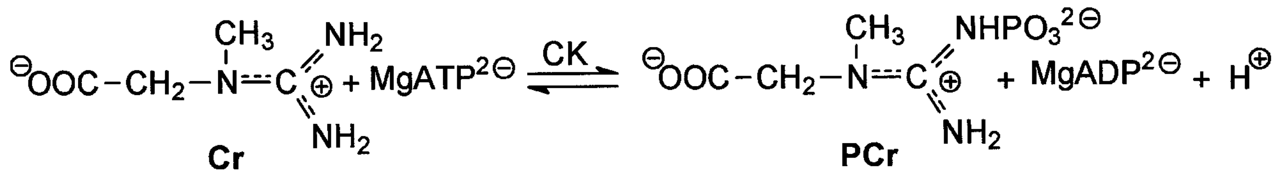


FIGURE 1 **Reaction catalyzed by creatine kinase.**

group from PCr (Lehmann, 1936). ATP, of course, is now known as the mediator of all energy changes within the cell.

There are four major CK isozymes, which have been named for the tissues from which they were historically isolated. The isozymes have been characterized on the basis of differences in gene and amino acid sequence, tissue localization and immunogenicity. There are two cytosolic forms, the muscle (MM-CK) and brain (BB-CK) forms, which exist as dimers under physiological conditions. Under some circumstances, cytosolic CK can exist as the MB heterodimer (Eppenberger *et al.*, 1967). Immunological studies show that antisera raised against MM-CK will not cross react with BB-CK, but there is cross reactivity with the same isozyme across several species (Chen *et al.*, 2000). There are also two mitochondrial forms of the enzyme, the ubiquitous (Mi_u-CK) and the sarcomeric (Mi_s-CK) forms which, based on their isoelectric point, are sometimes referred to as acidic (Mi_a-CK) and basic (Mi_b-CK) mitochondrial CK, respectively (Wyss *et al.*, 1992). The mitochondrial isoforms (MtCK) generally exist as octamers but can be readily dissociated into dimers (Wyss *et al.*, 1992).

For many years only the soluble form of creatine kinase was known, and the main physiological role ascribed to CK was the maintenance of energy homeostasis at sites of high energy turnover such as rapidly contracting skeletal muscle. The high levels of CK ensured that ADP and ATP levels remained almost constant, effectively buffering the cell against rapid depletion of ATP. The discovery of the mitochondrial isozymes showed that CK was located in individual "compartments" and the concept of a creatine-phosphocreatine shuttle was developed. Here distinct isozymes are associated with sites of ATP production and consumption, and they fulfil a role of a transport mechanism for high energy phosphates. Further discussion on the physiological role of CK, as well as the Cr-PCr shuttle, may be found in a special edition of *Molecular and Cellular Biochemistry* (Saks & Ventura-Clapier, 1994) as well as in the review by Wallimann *et al.* (1992).

Sequence Homology and Evolution

Creatine kinase is a member of the phosphagen (guanidino) kinase family. This family of enzymes is highly conserved and is found throughout the animal kingdom. Other members of the family include arginine kinase (AK), glycocyamine kinase (GK), taurocyamine kinase (TK), and lombricine kinase (LK). The structures of the various naturally occurring guanidino acceptors are shown in Figure 2. Creatine kinase is the only phosphagen kinase found in vertebrates, but it is also found in many invertebrates, including sponges, polychaetes, and echinoderms (Robin, 1964; Watts, 1968, 1971, 1975; Ellington, 2001). The phosphagen kinases are distributed along distinct phylogenetic and, sometimes, tissue-specific lines (Ellington, 2001). It has long been thought that AK is the most primitive phosphagen kinase and that the other members of the family arose by gene duplication followed by divergent evolution (Watts, 1971, 1975; Suzuki *et al.*, 1998). The fact that creatine kinase is present in sponges, the oldest of all multi-cellular animals, also suggests that the divergence of creatine kinase from an arginine kinase-like ancestral protein occurred very early (Sona *et al.*, 2004).

Four independent nuclear genes have been found to encode the individual isozymes of CK (Mühlebach *et al.*, 1994). Detailed analyses of the properties of these genes, including details of structure and regulation, can be found in reviews by Qin *et al.* (1998) and Suzuki *et al.* (2004). The full-length sequence of a mitochondrial isozyme is about 35 residues longer than that of its cytosolic counterpart. The additional residues belong to a leader peptide which is removed proteolytically, either during or after translocation across the mitochondrial membrane (Pfanner & Geissler, 2001). The mature gene products are between 40 and 44 kDa and, within each class of isozyme, amino acid sequence identities range from 85% to more than 99% (Mühlebach *et al.*, 1994; Qin *et al.*, 1998). The two cytosolic isozymes exhibit ca. 80% sequence identity, as do the two mitochondrial isozymes. However, the cytosolic and mitochondrial isozymes share only 60% to 65% sequence identity

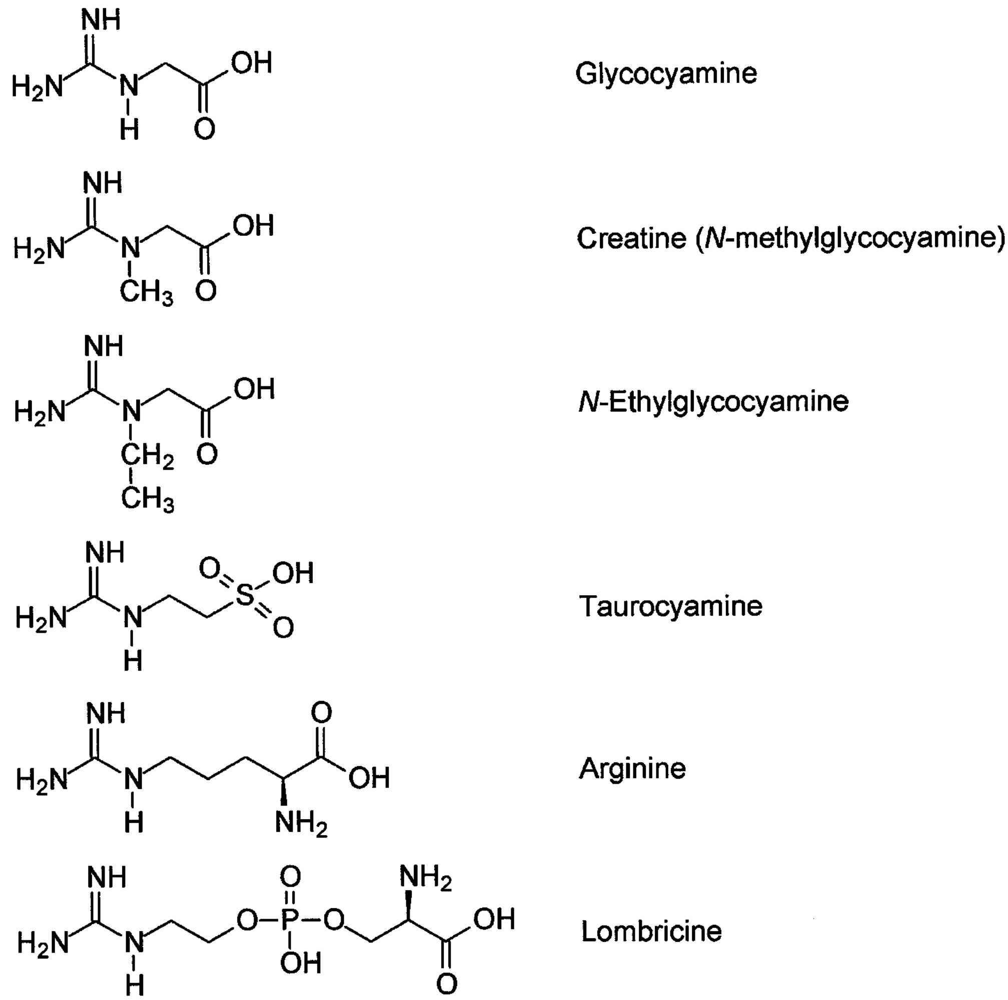


FIGURE 2 **Structures of naturally occurring substrates for phosphagen kinases.**

(Mühlebach *et al.*, 1994). Overall the sequence data confirm that the CKs are an evolutionarily conserved group of enzymes and suggest that the separation of the mitochondrial and cytosolic forms was due to a gene duplication event which occurred early in phylogeny. This, in turn, was followed by a second duplication event that gave rise to the two mitochondrial and two cytosolic isozymes (Mühlebach *et al.*, 1996; Qin *et al.*, 1998; Pineda & Ellington, 1999).

The amino acid sequences of the CK isozymes show six regions of extensive homology which are flanked by seven more variable regions (Mühlebach *et al.*, 1994). Consequently, there have been several studies aimed at linking regions of conserved sequence with function. For example, it was thought that the residues responsible for the membrane binding of mitochondrial CK were probably located in the C-terminal region of the protein (Fritz-Wolf *et al.*, 1996; Kabsch & Fritz-Wolf, 1997), a proposal that has been recently confirmed by Schlattner *et al.* (2004). The residues likely to be important for octamer formation, on the other hand, are located in the N-terminal region (Kaldis *et al.*, 1993, 1994). Of the two cytosolic isozymes it has been shown that only the muscle isoform interacts with the sarcomeric M-line. The interaction has been traced to two lysine pairs, which are highly conserved in MM-CK but which are not present in BB-CK (Hornemann *et al.*, 2000b). From a mechanistic standpoint, there are two significant regions of highly conserved sequence. These are the negatively charged NEED-box (Eder *et al.*, 2000b; Cantwell *et al.*, 2001) and the region surrounding a cysteine residue, which is highly susceptible to chemical modification (Kenyon & Reed, 1983; Furter *et al.*, 1993). Both of these regions are conserved across all phosphagen kinases and play a major role in the catalytic mechanism (*vide infra*).

In addition to the cytosolic and mitochondrial isozymes, another gene coding for a unique flagellar isoform (fCK), consisting of three fused creatine kinase domains, is found in many protostome and deuterostome

invertebrates and in a protochordate (Suzuki *et al.*, 2004). It is thought that the gene arose as a result of a duplication/fusion process followed by an unequal crossing over resulting in gene triplication (Wothe *et al.*, 1990). Phylogenetic analysis suggests that the flagellar isozyme is more closely linked to the cytosolic forms, but that the data are somewhat equivocal (Suzuki *et al.*, 2004). Given that no fCK has yet been characterized, it is included in this discussion only for the sake of completeness, and will not be referred to again.

PHYSICAL AND CATALYTIC PROPERTIES

The earliest detailed review of the creatine kinase literature was that of Kuby and Noltmann (1962). Ten years later, the review of Watts (1973) appeared, to be followed another decade later by that of Kenyon and Reed (1983). While these three reviews have summarized the majority of the early studies on CK, many of the salient points, and some of the more recent work, are described below.

Purification, Assay and Physical Properties

The first purification of CK (Kuby *et al.*, 1954) as well as the majority of the early studies of the physical properties and structure of creatine kinase were carried out on the enzyme isolated from rabbit muscle (RMCK). In the presence of divalent cations, at high ionic strength and low temperature, the enzyme could be separated from other proteins by fractionation with ethanol (Kuby *et al.*, 1954). This became the most commonly used isolation method, although development of affinity chromatography using Blue Sepharose followed by ion-exchange has removed the necessity for ethanol precipitation (Chen *et al.*, 2000).

As creatine kinase is of major clinical interest, particularly as a marker for myocardial infarction, there have been many methods developed for the assay of CK activity. The rate of creatine formation could be followed by reaction with an α-naphthol-diacetyl reagent (Eggleton *et al.*, 1943), whereas that of phosphocreatine formation was followed colorimetrically as the acid-molybdate-labile phosphate (Kuby *et al.*, 1954). These stop procedures were later replaced by continuous spectrophotometric methods using coupled enzyme systems. Reaction in the forward direction (production of phosphocreatine) may be followed at 340 nm by coupling to pyruvate kinase and lactate dehydrogenase as described by Tanzer and Gilvarg (1959). Conversely, ATP production (the reverse reaction) may also be followed at 340 nm by coupling to hexokinase and glucose-6-phosphate dehydrogenase as initially described by Oliver (1955) and later by Rosalki (1967). Another routinely used continuous assay, particularly for the forward reaction, is the pH-stat assay of Mahowald *et al.* (1962), which takes advantage of the fact that a proton is generated when phosphocreatine is produced (Figure 1). One advantage of the latter method is that it shows less interference from other enzymatic activities and, consequently, can be used with crude enzyme preparations. However, if this assay is used for the reverse reaction it should be noted that, at pH values below 6.0, PCr transphosphorylation and H^+ consumption are no longer equimolar and pH-stat values must be corrected accordingly (Furter *et al.*, 1993).

Early sedimentation velocity studies suggested a molecular weight of 81,000, and that the molecule behaved as an unhydrated ellipsoid (Kuby & Noltmann, 1962). Later it was shown that creatine kinase consisted of two readily dissociable subunits, with no disulfide bridges, and a molecular weight of 82,600 Da. For other species, the molecular weight varied between 78,500 and 85,100 Da. Further sedimentation studies showed that denaturation with guanidinium chloride first dissociated and then unfolded the subunits into random coil configuration, whereas sodium dodecyl sulfate caused them to dissociate without any apparent loss of structural organization. Overall it was concluded that the enzyme comprised two cigar-shaped subunits lying side by side (Watts, 1973).

Although the cytosolic isozymes are found as dimers, their mitochondrial counterparts are generally octameric. However, when placed in a 'transition-state analog complex' (TSAC) mixture comprised of creatine, MgADP and planar anions such as nitrate, nitrite and formate (Milner-White & Watts, 1971), octameric Mi_b-CK dissociates into dimers (Gross & Wallimann, 1993). Consequently it appeared that the minimal catalytically active form of CK was the dimer, but this remained the subject of some conjecture.

Another of the continuing debates relates to the role of the individual subunits in catalysis. The early studies on the CK subunits, reviewed by Bickerstaff and Price (1978), focused on the behavior of the isolated

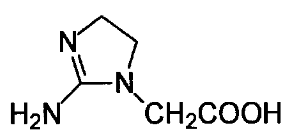
subunits. They established that there was a single active site per subunit, that the active sites were well separated, and further, that there was some indication that the monomeric form of the enzyme was active. Several studies examined the possible non-identical behavior of the subunits with evidence being provided to show that CK possesses either negative cooperativity or non-identical active sites (Price & Hunter, 1976; Bickerstaff & Price, 1978). Nevinsky *et al.* (1982), for example, showed that the subunits of rabbit muscle CK were functionally non-identical, whereas Degani and Degani (1979) suggested that the subunits were arranged asymmetrically and that a dimer was required for activity. More recent studies have demonstrated that the subunits act either independently (Wang *et al.*, 1990) or independently in the forward direction and cooperatively in the reverse reaction (Hornemann *et al.*, 2000a). In addition, a kinetic analysis has indicated that the two subunits may adopt different tertiary structures and behave as distinct entities (Wang & Pan, 1996).

There was an expectation that this question would be conclusively answered when the X-ray structure of substrate-bound creatine kinase became available. At first glance it was, with the crystallographic asymmetric unit of *Torpedo californica* CK (TcCK) found to contain two monomers that were not identical in either conformation or ligand binding state (Lahiri *et al.*, 2002). This enzyme was crystallized in the presence of a TSAC mixture comprised of MgADP, creatine and nitrate at concentrations well above the K_i of the TSAC. Each biological dimer was found to contain one monomer bound to MgADP and a second monomer bound to the TSAC (Lahiri *et al.*, 2002). Certainly this was suggestive of negative cooperativity yet, almost immediately, a mass spectrometric hydrogen/deuterium exchange experiment demonstrated conclusively that, in solution, both subunits of a dimer can bind substrates (Mazon *et al.*, 2003).

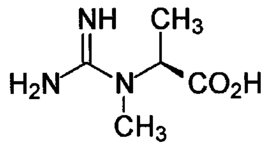
What has become clear is that an individual subunit of CK can catalyze the transphosphorylation reaction. This results from a study by Cox *et al.* (2003) in which site-directed mutagenesis was employed to remove several interactions at the dimer interface of RMCK. Several mutants were expressed in a soluble form and were purified by affinity chromatography. Size-exclusion chromatography and analytical centrifugation indicated that, at 1 mg/mL, two of them were monomeric. Kinetic analysis demonstrated that both these mutants were active for reaction in the direction of phosphocreatine synthesis, but the K_d values for both creatine and MgATP were about an order of magnitude higher than those of the WT enzyme. In addition, the data for the one of the mutants could only be fitted to the equation for an ordered mechanism wherein creatine binds first (Cox *et al.*, 2003). These experiments provide an unequivocal demonstration that, like the lobster arginine kinase, which catalyzes an analogous phosphorylation reaction in invertebrates (Morrison, 1973), creatine kinase can be active as a monomer.

Substrate Specificity

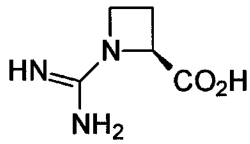
Creatine kinase possesses a very narrow substrate specificity. Of the naturally occurring substrates for guanidino kinases (Figure 2), only creatine (N-methylglycocyamine), glycocyamine (Tanzer & Gilvarg, 1959) and N-ethylglycocyamine (Ennor *et al.*, 1955) were found to be substrates. The list of substrates was later expanded (Figure 3) to include compounds such as cyclocreatine (1-carboxymethyl-2-iminioimidazolidine) and (*R*)-*N*-methyl-*N*-amidino-β-alanine (Rowley *et al.*, 1971). Cyclocreatine, in particular, proved to be useful in showing that creatine is phosphorylated on the nitrogen *trans* to the methyl group (Rowley *et al.*, 1971; Struve *et al.*, 1977), while other alternative substrates were useful in showing that the active site can tolerate only limited steric bulk, and that the planarity and orientation of the guanidino group is important for catalysis (McLaughlin *et al.*, 1972). More recently, Boehm *et al.* (1996) established that, when a porcine carotid artery, which contains a high percentage of cytosolic CK, was perfused with cyclocreatine, guanidinoacetic acid (glycocyamine), β-guanidinopropinic acid (Figure 3) or *N*-methylguanidinopropionic acid (Figure 3), the phosphorylated analogue was accumulated in all cases. Furthermore, all the analog could be dephosphorylated, albeit relatively slowly in the case of phosphocyclocreatine. Overall the results suggested that a poor *in vitro* substrate for cytosolic CK could be utilized effectively *in vivo* (Boehm *et al.*, 1996). Conversely, they found that the mitochondrial CK in intact mitochondria could only phosphorylate creatine and cyclocreatine, suggesting that MtCK may have a greater specificity than its cytosolic counterpart (Boehm *et al.*, 1996). Although the nucleotide triphosphate and nucleotide diphosphate are essential for the forward and reverse reactions, respectively, James and Morrison (1966) showed that ADP



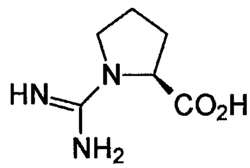
1-Carboxymethyl-2-iminioimidazolidine (cyclocreatine)



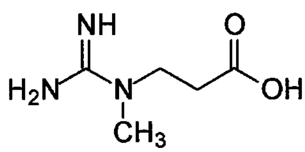
(*R*)-*N*-Methyl-*N*-amidinoalanine



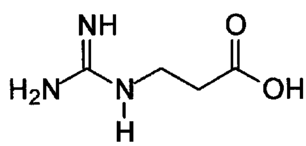
(*R*)-*N*-Amidinoazetidine-2-carboxylic acid



(*R*)-*N*-Amidinoproline



N-Methylguanidinopropionic acid



β-Guanidinopropionic acid

FIGURE 3 **Structures of alternative substrates for phosphagen kinases.**

could be effectively replaced by dADP. In fact, the rabbit muscle isozyme showed a broad specificity for the base (James & Morrison, 1966) that was not reflected in BB-CK and MM-CK from other species (Watts, 1973). It is also noteworthy that, in addition to Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+} have been used as activators whereas Ni^{2+}, Cr^{2+}, and Cd^{2+} are either inactive or inhibitory (O'Sullivan & Morrison, 1963). Optimal activity was obtained when Mg^{2+}, provided as the acetate, was added in 1 mM excess over the nucleotide concentration (Cleland, 1967), suggesting that MgATP and MgADP were the true substrates for the reaction.

Kinetic Analyses

The initial kinetic studies undertaken on the rabbit muscle isozyme are summarized in Figure 4. The overall reaction is reversible and, at pH 8.0 and above, the enzyme operates by a rapid equilibrium random bimolecular, bimolecular mechanism (Figure 4A). No evidence has been obtained for any phosphorylated enzyme intermediate, and phosphoryl transfer was found to be the rate-determining step (Morrison & James, 1965; Morrison & Cleland, 1966; Morrison & White, 1967). At pH 7.0 and below, the situation is more complex (Schimerlik & Cleland, 1973). In the forward (creatine phosphorylation) direction, RMCK operates by an equilibrium ordered mechanism with ATP binding before creatine (Figure 4B), while in the reverse direction the reaction remains random (Schimerlik & Cleland, 1973).

Although there have been numerous measurements of kinetic constants for the soluble isozymes, data for the mitochondrial isozymes, particularly the ubiquitous

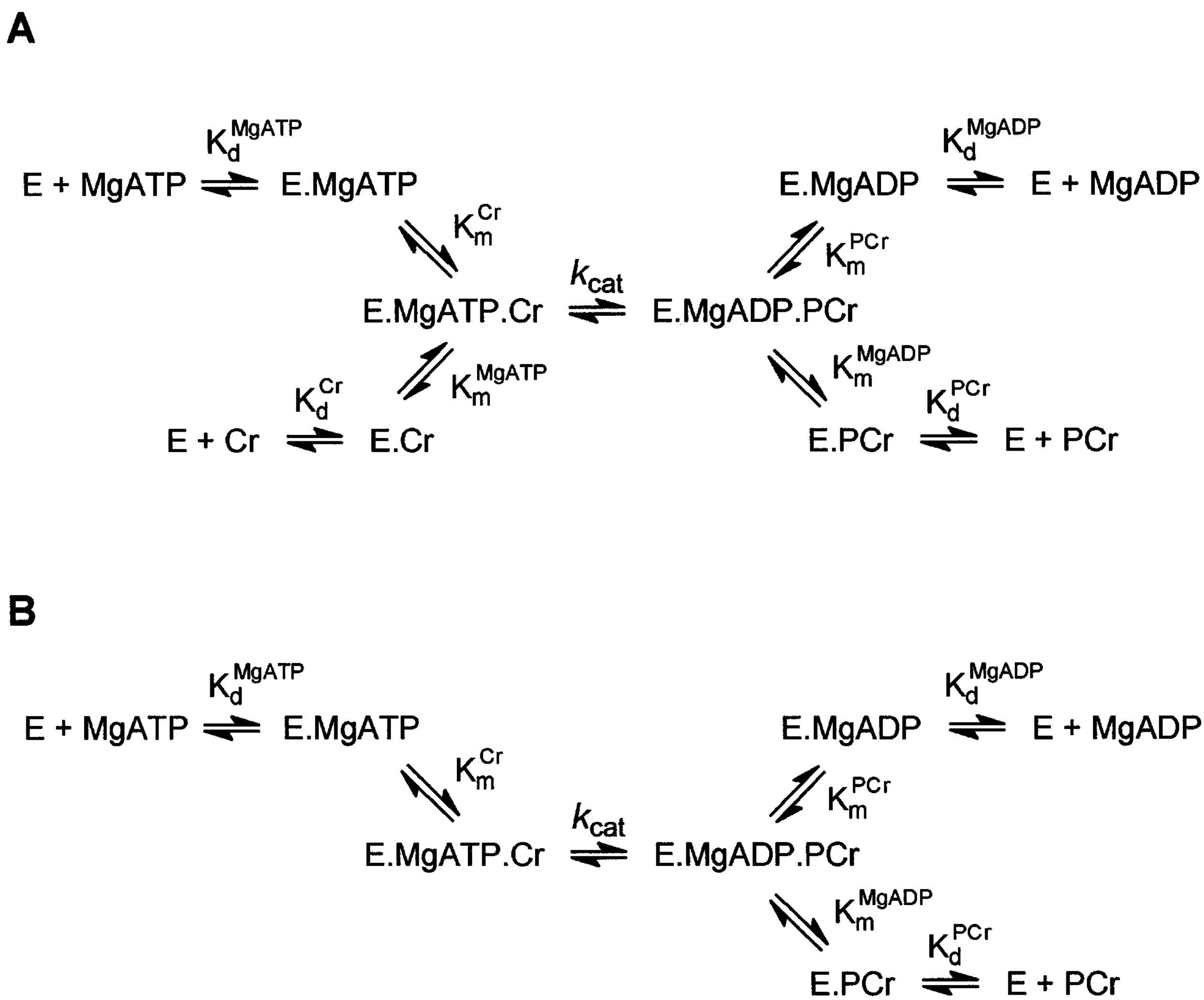


FIGURE 4 Kinetic mechanism of rabbit muscle CK at (A) pH 8.0 and above (Morrison & James, 1965) and (B) pH 7.0 and below (Schimerlik & Cleland, 1973).

isozyme, are scarce (Schlattner *et al.*, 2000). Specific activities for the muscle isozymes were similar across a number of mammalian species and not dissimilar to their brain counterparts (Watts, 1973). Tables 1 and 2 provide a comparison of the kinetic data for all four human isozymes in both the forward (phosphocreatine production) and reverse (ATP production) directions. The mitochondrial isozymes (MtCK) are generally 3 to 4 times slower than their cytosolic counterparts, and the reverse reaction is always faster than the forward reaction, with values of k_{cat}(for)/k_{cat}(rev) ranging from 0.32 (HMCK) to 0.85 (sMtCK). With the exception

TABLE 1 **Kinetic constants for reaction in the forward direction (*i.e.*, creatine phosphorylation).**

	HMCK[a]	HBCK[a]	uMtCK[b]	sMtCK[b]
k_{cat} (min^{-1})	9.21×10^3	12.9×10^3	3.1×10^3	4.5×10^3
K_d Cr (mM)	14.6 ± 1.2	6.0 ± 0.3	45.8 ± 11.2	43.9 ± 14.9
K_m Cr (mM)	9.5 ± 0.59	4.9 ± 0.4	1.01 ± 0.13	7.31 ± 1.27
k_{cat}/K_m Cr (mM/min^{-1})	9.7×10^2	2.6×10^3	3.1×10^3	6.2×10^2
K_d MgATP (mM)	1.2 ± 0.11	0.99 ± 0.05	4.04 ± 1.33	4.06 ± 0.78
K_m MgATP (mM)	0.89 ± 0.16	0.81 ± 0.10	0.11 ± 0.02	0.68 ± 0.21
k_{cat}/K_m MgATP (mM/min^{-1})	1.0×10^4	1.6×10^4	2.8×10^4	6.6×10^3

[a]From (Chen *et al.*, 2000). Reaction was at pH 9.0.
[b]From (Schlattner *et al.*, 2000). Reaction was at pH 8.0.

TABLE 2 Kinetic constants for reaction at pH 7.0 in the reverse reaction (*i.e.*, ATP production).

	HMCK[a,b]	HBCK[b]	uMtCK[c]	sMtCK[c]
k_{cat} (min^{-1})	2.9×10^4	2.1×10^4	4.7×10^3	5.4×10^3
K_d PCr (mM)	3.7 ± 1.0	0.22 ± 0.05	0.92 ± 0.13	2.87 ± 0.68
K_m PCr (mM)	1.33 ± 0.14	0.51 ± 0.06	0.55 ± 0.03	1.16 ± 0.14
k_{cat}/K_m PCr (mM/min^{-1})	2.2×10^4	4.1×10^4	8.6×10^3	4.7×10^3
K_d MgADP (mM)	0.07 ± 0.013	0.02 ± 0.002	0.22 ± 0.03	0.38 ± 0.09
K_m MgADP (mM)	0.03 ± 0.005	0.04 ± 0.002	0.13 ± 0.01	0.15 ± 0.02
k_{cat}/K_m MgADP (mM/min^{-1})	1.1×10^6	5.3×10^5	3.6×10^4	3.6×10^4

[a]From (Wang *et al.*, 2001).
[b]Wang, McLeish, Kenyon (unpublished results). Results are reported as ± S.E.M.
[c]From (Schlattner *et al.*, 2000).

of the HBCK in the reverse reaction, K_m values were significantly lower than K_d values, where K_m and K_d are the dissociation constants from the ternary and the binary complexes, respectively (Figure 4). A ratio of K_m/K_d (α-value) of less than one is indicative of substrate synergy wherein the binding of one substrate increases the affinity for the second substrate (Segel, 1975). At this time it is unclear why the reverse reaction with HBCK has an α-value of greater than unity but the degree of synergism in creatine kinases has been known to vary with reaction conditions (Morrison & James, 1965; Maggio *et al.*, 1977) and can also be affected by mutagenesis (Novak *et al.*, 2004). Overall the α-values were reasonably similar, the major exception being for uMtCK in the forward reaction where its α-value was almost an order of magnitude lower than those of the other isozymes, a discrepancy that was also manifest in the K_m values for both creatine and ATP. The value of k_{cat}/K_m is often used as a measure of the specificity of an enzyme for its substrates (Copeland, 2000). Tables 1 and 2 show that the sMtCK has the lowest specificity for any of its substrates and that the cytosolic isozymes are generally more efficient than the mitochondrial isozymes. This variation is more pronounced in the reverse reaction, where there is a ten-fold difference between the cytosolic and mitochondrial enzymes. At this point it is unclear whether the catalytic differences between the various isozymes have any important physiological significance.

In addition to the Michaelis-Menten kinetics there have been several determinations of the equilibrium constant for the reaction. These show that the equilibrium lies to the right (*i.e.*, favors PCr formation) and that the equilibrium constant greatly depends on both the concentration of free magnesium ions and the pH (Kuby & Noltmann, 1962; Watts, 1973; Lawson & Veech, 1979; Lerman & Cohn, 1980; Huddleston *et al.*, 1994).

Both sulfate and phosphate anions have been found to inhibit CK, competitively with respect to ATP and phosphocreatine and noncompetitively with respect to ADP and creatine. Based on these results, it was thought that small anions would occupy the same site on the enzyme as the transferring phosphoryl group, and their effectiveness as an inhibitor would be based on the extent to which they mimicked the phosphate group (Nihei *et al.*, 1961; Kumudavalli *et al.*, 1970). However, kinetic analyses showed that anions affected the initial velocities and the shape of the progress curves in a manner incompatible with that proposal (Milner-White & Watts, 1971). In addition there were reports that an equilibrium mixture of substrates protected the enzyme against alkylation by iodoacetamide (Watts & Rabin, 1962) and that a 'dead-end complex' of creatine-MgADP afforded even greater protection (O'Sullivan *et al.*, 1966). In a study that was alluded to briefly earlier, Milner-White and Watts (1971) showed that an anion was involved in addition to the substrates, that the level of substrate protection depended on the type of anion present, and that a planar anion or a halide was required for maximum inhibition. They noted that the CK reaction potentially involves a direct transfer of a phosphoryl group by an S_N2 type reaction, with the phosphoryl group forming an sp^3d hybrid in the transition state. On that basis they proposed that planar anions, such as nitrite and formate, mimicked the phosphoryl group in the transition state of the reaction and suggested that a quaternary complex (E–TSAC) comprising enzyme–creatine–anion–MgADP was formed (Milner-White & Watts, 1971). This study and its predictions, their accuracy confirmed by later spectroscopic (Reed & Cohn, 1972; Reed & Leyh, 1980) and X-ray (Lahiri *et al.*,

2002) experiments, not only addressed and explained the anomalous kinetic results, but also provided an experimental tool that would be used in a variety of subsequent investigations. It should also be noted that, from an entropic standpoint, it is much easier for the enzyme to bind its two substrates than to assemble the three components of the TSAC. Recently, Borders *et al.* (2002) used intrinsic fluorescence measurements to determine a dissociation constant of 4×10^{-10} M^3 for the TSAC into its individual components. This was possibly the first report of the dissociation constant for a ternary, let alone a quaternary, transition state analog complex.

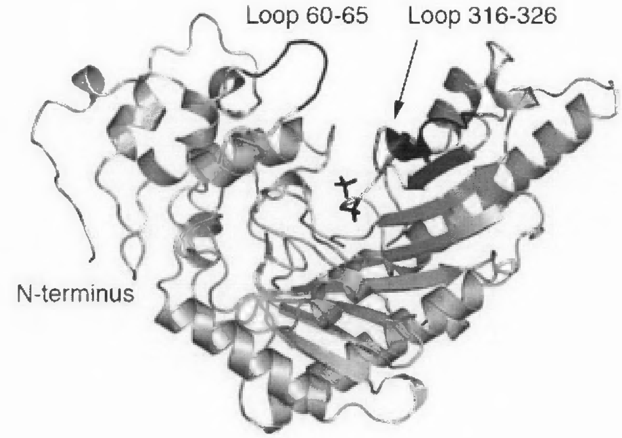


FIGURE 5 Ribbon diagram of sarcomeric chicken creatine kinase (Mi_b-CK) monomer. The two flexible loops are highlighted. The ATP binding site is identified by the two phosphates in the cleft between the N-and C-terminal domains. Coordinates used were those from PDB 1CRK (Fritz-Wolf *et al.*, 1996).

THREE DIMENSIONAL (X-RAY) STRUCTURE

Almost since it was first isolated, attempts have been made to crystallize creatine kinase (Kuby *et al.*, 1954). Both the cytosolic (Keutel *et al.*, 1972; McPherson, 1973; Burgess *et al.*, 1978; Takasawa *et al.*, 1981; Gilliland *et al.*, 1983; Hershenson *et al.*, 1986) and mitochondrial (Schnyder *et al.*, 1990, 1991) isozymes have proved to be amenable to crystallization. However, although preliminary X-ray measurements on CK had been carried out by 1973 (McPherson, 1973), it was another two decades before the first X-ray structure of creatine kinase was published.

The Structure of Sarcomeric Mitochondrial Creatine Kinase (Mi_b-CK)

In 1996, Fritz-Wolf *et al.* reported the structure of the sarcomeric chicken cardiac Mi_b-CK, in the presence and absence of NaATP, at 3 Å resolution (Fritz-Wolf *et al.*, 1996). There were four crystallographically independent monomers with virtually identical structures. Each monomer consisted of a small (residues 1 to 112) N-terminal domain and a larger (residues 113 to 380) C-terminal domain, with the ATP binding site being located in a cleft between the two domains (Figure 5). In addition to the five N-terminal residues, two loops, comprising residues 60 to 65 and 316 to 326, were shown to be highly flexible. Small-angle X-ray scattering experiments (Forstner *et al.*, 1996) had demonstrated that Mi_b-CK exhibits a considerable decrease in the radius of gyration in the presence of MgATP or a TSAC. Conversely neither ATP nor ADP alone had any effect on the scattering curves. Given the position of the flexible loops it was postulated that, when creatine and a magnesium ion were bound, these loops would move to close over the active site and exclude water during catalysis (Fritz-Wolf *et al.*, 1996; Kabsch & Fritz-Wolf, 1997).

As expected, the six conserved regions of high sequence homology (Mühlebach *et al.*, 1994) were found to form the core of the structure, covering the active site of the enzyme. The active site itself was found to contain many of the residues implicated by earlier studies as being either located in the active site or important catalytically. Nuclear magnetic resonance (NMR) studies, for example, had shown that four histidine residues were located within 18 Å of the Cr^{3+} of a Cr^{3+}-ATP complex bound to the enzyme (Rosevear *et al.*, 1981). One of these histidines was also postulated to have a $pK_a \sim 7$, and to act as an acid/base catalyst (Cook *et al.*, 1981). However, none of the histidines was within 12 Å of the γ-phosphate of ATP and, intriguingly, mutagenesis of each of these histidine residues showed that none were essential for catalysis (Chen *et al.*, 1996; Forstner *et al.*, 1997). The pH studies which implicated the histidine residue also indicated that an ionized carboxylic acid group was required for the binding of both creatine and phosphocreatine (Cook *et al.*, 1981). The X-ray structure of Mi_b-CK showed that two glutamic acid residues, Glu226 and Glu227, as well as an aspartic acid residue, Asp228, were located in the active site, any of which could fulfil this role. Note that, unless stated

otherwise, the numbering of residues is that of the CK isozyme under immediate discussion.

Chemical modification studies had shown that rabbit muscle CK was fully inactivated by the arginine specific reagent, phenylglyoxal (Borders & Riordan, 1975), whereas NMR had shown that an arginine residue was located near the ATP binding site (James, 1976). Consequently it was not surprising when the X-ray structure revealed that the phosphate group of ATP interacted with four arginine residues and that two more arginine residues are within 5 Å of the γ-phosphate. Also, not unexpectedly, a tryptophan residue was found in the active site. This residue, Trp223, was deduced by earlier NMR and fluorescence measurements (Vasák *et al.*, 1979; Messmer & Kagi, 1985), as well as by mutagenesis studies (Gross *et al.*, 1994), as being important for catalysis.

Over the years, there have been numerous studies implicating a cysteine residue in the CK active site. In both the cytosolic (Mahowald *et al.*, 1962) and mitochondrial (Fedosov & Belousova, 1988; Wyss *et al.*, 1993) isoforms, one highly reactive cysteine residue per monomer is able to react with sulfhydryl-specific reagents. Marletta and Kenyon (1979) used the creatine-based affinity label, epoxycreatine, to completely inactivate rabbit muscle CK, thereby confirming that the reactive group, possibly a cysteine or a carboxylate, must be located adjacent to the creatine binding site. Subsequently, tryptic digestion combined with mass spectrometry was used to show that it was a cysteine residue, identified as Cys282, that was labeled (Buechter *et al.*, 1992). This was one of the two fully conserved cysteine residues in the CKs, and the only cysteine residue to be conserved across all guanidino kinases. The X-ray structure of Mi_b-CK showed that Cys278 (analogous to Cys282 in RMCK) was indeed located within the active site and was positioned near the γ-phosphate of ATP and near the acidic residues thought to be involved in creatine binding.

Although the well-conserved residues formed a compact core containing the active site, it was possible also to identify residues in the less-conserved regions of sequence that could be linked to isoform specific properties. For example, each monomer was shown to contain four contact regions, two of which could be linked to octamer formation, and three to dimer formation. A detailed analysis of the structure and its relationship to the cellular functions of creatine kinase may be found in a review by Schlattner *et al.* (1998).

Comparison of Structures from All Four Isozymes

Following the publication of the Mi_b-CK structure (Fritz-Wolf *et al.*, 1996) the X-ray structures of the rabbit muscle isozyme (Rao *et al.*, 1998), the cytosolic chicken brain (BB-CK) isozyme (Eder *et al.*, 1999), the human ubiquitous mitochondrial (Mi_a-CK) isozyme (Eder *et al.*, 2000a), the bovine muscle (MM-CK) isozyme (Tisi *et al.*, 2001), and the human muscle (MM-CK) isozyme (Shen *et al.*, 2001) followed in rapid succession. Not surprisingly, given the level of sequence identity, the overall structures were all very similar, particularly in the region of the active site. Nonetheless, comparison of these structures was able to provide a rationale for some of the functional differences observed among the various CK isozymes.

Amino acid sequence alignments had suggested that isozyme-specific sequences were localized at the N- and C-termini, the linker region between two domains, and in parts of three helices. The structural comparisons showed that, with the exception of the N-terminal region, the three dimensional structure was conserved in the regions of divergent sequence although there were changes in the electrostatic surface of the protein. Before any CK structures were available, it had been predicted that the residues likely to be important for octamer formation would be located in the N-terminal region (Kaldis *et al.*, 1993, 1994). Comparison of the X-ray structures of the two cytosolic BB-CK isoforms (Eder *et al.*, 1999; Tisi *et al.*, 2001) showed that the N-terminal region was important for dimer formation. It was also found to be important for octamer formation in the mitochondrial isoforms (Fritz-Wolf *et al.*, 1996; Eder *et al.*, 2000a) but, unfortunately, the seven N-terminal amino acids are disordered in the structures of the MM-CK isoforms (Rao *et al.*, 1998; Shen *et al.*, 2001). However, there are still significant differences between the human muscle isoform residues 8 to 15 and residues 1 to 10 of the human mitochondrial isoforms which are presumably related to octamer formation (Shen *et al.*, 2001). It is also interesting to note that the contact surface area between monomers in a chicken BB-CK dimer is almost 50% greater than the contact area between two MtCK monomers, presumably reflecting the greater stability of the dimer in the cytosolic isoforms (Eder *et al.*, 1999, 2000a). In addition, there are differences in the stability of the octamers that can also be related to contact surface areas, this time

of the dimer-dimer interface. For example, the human Mi_a-CK isoform forms a more stable octamer than the Mi_b-CK and has more than twice the contact surface between its dimers (Eder *et al.*, 2000a). It must also be noted that, although these N-terminal interactions are important for monomer-monomer and dimer-dimer interactions, other regions of the structure are of equal or greater import (Fritz-Wolf *et al.*, 1996). Finally, the C-terminal region of the mitochondrial isozymes contains 3 to 4 additional residues, two of which are positively charged. At present it is unclear whether the CK octamer binds only to the membrane surface (Cheneval *et al.*, 1989) or enters the lipid bilayer (Rojo *et al.*, 1991; Vacheron *et al.*, 1997). It has been postulated that these charged groups may bind to the negatively charged head groups of the cardiolipin of the mitochondrial membrane (Fritz-Wolf *et al.*, 1996; Eder *et al.*, 1999), but it has also been suggested that the predominantly hydrophobic character of the five C-terminal residues of mitochondrial CKs are also well suited for membrane insertion (Eder *et al.*, 2000a).

Whereas these structures were useful in that they identified many active site residues and provided significant information on those residues involved in dimer and octamer formation, they were of less use in attempts to relate structure with mechanism. The foremost problem was that, with the exception of that of the Mi_b-CK isoform, none of these structures was obtained in the presence of either substrates or inhibitors. Indeed, even the structure of Mi_b-CK liganded to ATP was obtained in the absence of Mg^{2+}, and each ATP molecule was found at a slightly different position in its monomer (Fritz-Wolf *et al.*, 1996). *In toto*, the structures provided no details about creatine binding, nor did they provide details about the binding of any substrate in the ternary enzyme complex. This was a serious limitation in that EPR (McLaughlin *et al.*, 1976) and small angle X-ray scattering (Forstner *et al.*, 1996, 1998) studies, as well as later infrared difference (Granjon *et al.*, 2001) and hydrogen-deuterium exchange (Mazon *et al.*, 2003) experiments, have all indicated that substrate binding brings about a considerable conformational change in CK. As alluded to earlier, small-angle X-ray scattering experiments had demonstrated that CK, in the presence of MgATP and the TSAC mixture, shows a decrease in radius of gyration (Forstner *et al.*, 1996; Forstner *et al.*, 1998). This decrease was similar to that observed for the closely related enzyme, arginine kinase, in the presence of MgADP or MgATP (Dumas & Janin, 1983; Forstner *et al.*, 1998). In AK it was proposed that this decrease was consistent with the hinge rotation of two domains (Dumas & Janin, 1983). Later comparison of the X-ray structure of the substrate-free AK (Yousef *et al.*, 2003) with the structure of AK liganded to a TSAC comprising arginine, nitrate and MgADP (Zhou *et al.*, 1998) confirmed the domain movement.

X-Ray Structures of Arginine Kinase

The AK-TSAC structure, initially reported at 1.86 Å resolution (Zhou *et al.*, 1998) and now refined to 1.2 Å (Yousef *et al.*, 2002), provided an immediate boost to the study of CK. Arginine kinase and creatine kinase share a relatively high level (38% to 44%) of sequence identity (Mühlebach *et al.*, 1994) and have almost superimposable far-UV CD spectra (Oriol & Landon, 1970; Mühlebach *et al.*, 1994). As with CK, AK possesses a reactive active site cysteine residue (Der Terrossian *et al.*, 1969) and has similar overall kinetic properties (Morrison, 1973). Now it was possible to compare the various CK structures with that of the AK-TSAC structure (Zhou *et al.*, 1998) and confirm that there was considerable structural homology as well. Given those similarities it was not unreasonable to model the transition-state conformation of CK by superimposing the structures of the "open" (substrate-free) forms of CK on the "closed" AK-TSAC structure (Eder *et al.*, 1999; Zhou *et al.*, 2000). The superimposition identified two flexible loops that would presumably undergo large conformational changes during catalysis. These were the loops containing residues 60 to 65 and 316 to 326 (Figure 5) which were characterized by either high temperature (B-) factors or were disordered. This model of the closed form of the enzyme was sufficient to direct a number of mutagenesis/mechanistic studies on CK (Eder *et al.*, 2000b; Cantwell *et al.*, 2001; Wang *et al.*, 2001), as well as on AK itself (Pruett *et al.*, 2003; Azzi *et al.*, 2004; Gattis *et al.*, 2004; Uda & Suzuki, 2004). Nonetheless, the precise details of the involvement of individual CK residues in particularly substrate binding and specificity remained elusive.

Structure of Substrate-Bound Creatine Kinase and Correlation with Mechanistic Studies

Recently, the X-ray structure of *Torpedo californica* creatine kinase (TcCK), a MM-CK isozyme, liganded

to a TSAC complex of creatine-nitrate-MgADP, was published (Lahiri *et al.*, 2002). As expected from the sequence similarity, TcCK shows considerable overall fold similarity with the other known creatine kinase structures. Fortuitously, a structure of TcCK bound to MgADP was also obtained from the same crystal (Lahiri *et al.*, 2002), which permitted a direct comparison of the open (CK-MgADP) and closed (CK-TSAC) forms of the enzyme. As shown in Figure 6, the residues involved in nucleotide binding did not move greatly on going from the open to the closed form. However, two residues, Ile69 and Val325, moved into the active site

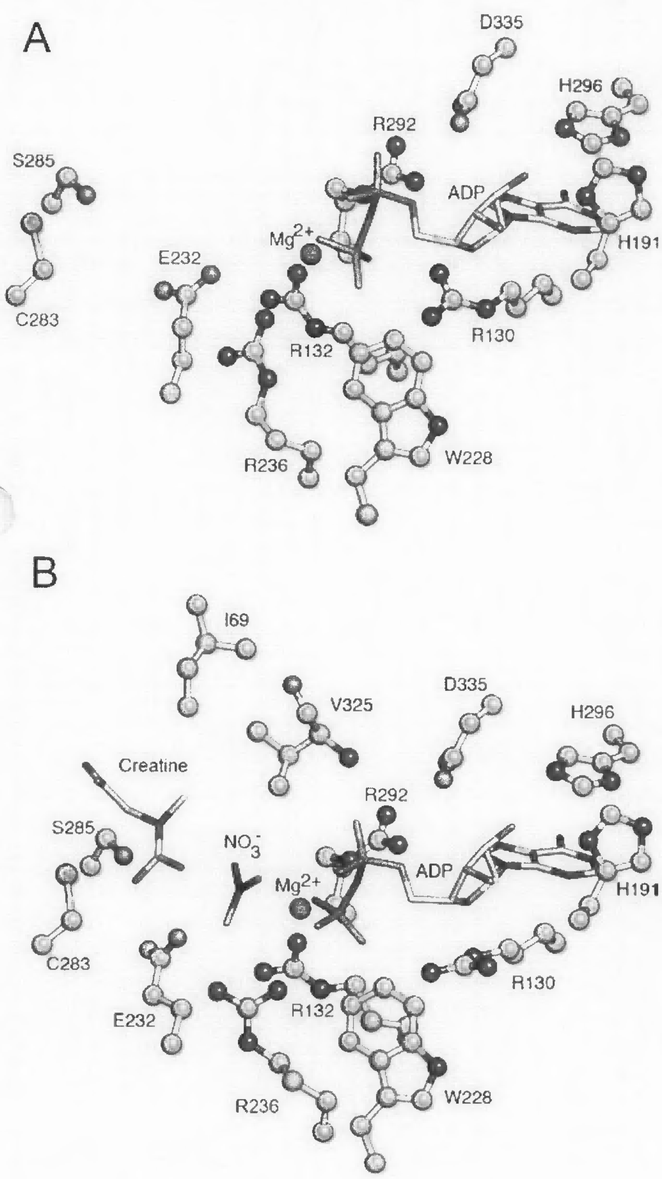


FIGURE 6 Active site of *Torpedo californica* creatine kinase bound to (A) MgADP and (B) a TSAC comprised of creatine, NO_3^- and MgADP. For clarity, the water molecules bound to Mg^{2+} and Arg320 which interacts with the phosphates of MgADP are omitted. Coordinates used were those from PDB 1N16 (Lahiri *et al.*, 2002).

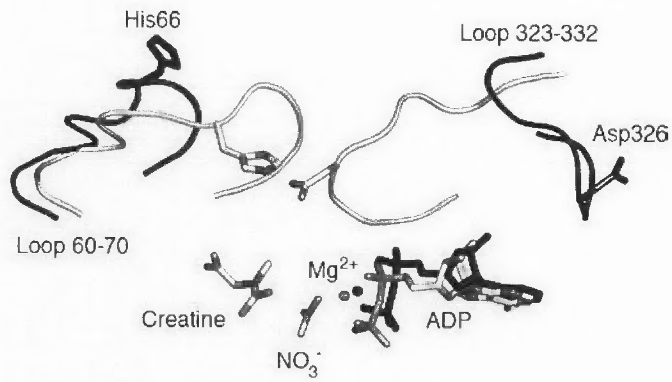


FIGURE 7 Overlay of the structures of *Torpedo californica* creatine kinase bound to MgADP (dark) and to the TSAC (light). The movement of His66 and Asp326 are highlighted. More than 25 Å apart in the MgADP-liganded enzyme, they approach to within 3 Å in the TSAC structure, forming a "latch" over the active site. Conversely, there is little movement of either the Mg^{2+} ion, or ADP. Again, the coordinates used were those from PDB 1N16 (Lahiri *et al.*, 2002).

possibly to provide a binding pocket for the N-methyl group of creatine. These two residues were brought into place by the movement of the flexible loops as they closed over the active site in the transition state. The loops also brought together His66 and Asp326, which appear to form a "latch" locking the flexible loops in place (Figure 7). Concomitantly, the flexible loops become more ordered with B-factors similar to those of the average main chain B-factor (Lahiri *et al.*, 2002).

It is somewhat intriguing that, in the same biological dimer, open and closed complexes can be observed. The fact that the TcCK-MgADP complex is in an open conformation is even more surprising, given that small-angle X-ray scattering showed a decreased radius of gyration when MgATP was added to CK (Forstner *et al.*, 1996; Forstner *et al.*, 1998) or when MgADP was added to AK (Dumas & Janin, 1983). It is conceivable that crystal packing forces might constrain asymmetrically the conformation changes required for substrate binding, thus giving rise to differences between the subunits in the crystal. Such a rationale is necessary as hydrogen-deuterium exchange experiments demonstrated that, in the presence of a TSAC mixture, both subunits of a dimer can bind substrates (Mazon *et al.*, 2003).

The Nucleotide Binding Site

In both complexes the MgADP groups are virtually superimposable, indicating that there is little or no strain involved in catalysis (Figures 6 and 7). As predicted by previous NMR experiments (Rosevear *et al.*, 1981) the adenine base in the ADP is in the *anti*

conformation with respect to the ribose ring, and the adenosine rings are held in place by stacking interactions with His296 and hydrogen bonds to His191 as well as to the main chain and to a few water molecules. His296 has been demonstrated to be particularly important by studies showing that even the conservative replacement with asparagine resulted in a considerable decrease in both substrate binding affinity and catalytic activity (Chen *et al.*, 1996).

As described earlier for the Mi_b-CK isozyme, the nucleotide phosphate binding pocket is formed primarily by five highly conserved arginines (Arg130, Arg132, Arg236, Arg292, and Arg320) that stabilize the negatively charged nonbonding oxygens of phosphate groups by a series of monodentate and bidentate interactions. The arginines are in the same position in both the CK-TSAC and CK-MgADP structures, except Arg320. In the E-MgADP structure, this residue interacts only with the nonbonded oxygen of the α-phosphate, but, in the E-TSAC complex, Arg320 moves closer and changes to bidentate ligand geometry, forming an additional hydrogen bond to an oxygen of the nitrate group. It is conceivable that Arg320, which is located at the base of one of the flexible loops, may be involved in conformational switching (Forstner *et al.*, 1998). In addition to the arginine residues Trp228 must form part of the binding pocket for, although it does not interact directly with the substrate, mutagenesis of this residue leads to inactivation of the enzyme (Gross *et al.*, 1994).

The Metal Ion

Cleland (1967) suggested that MgATP and MgADP were the true substrates for the reaction. Exchange-inert Cr(III) nucleotide complexes were initially used to determine the stereochemistry of the interaction with CK (Dunaway-Mariano & Cleland, 1980), and a mechanism was proposed in which the Mg(II) has β,γ-coordination to ATP prior to phosphoryl transfer and then migrates to form an α,β-bidentate complex with the product, ADP. A later study suggested that α,β,γ-coordination to ATP is also feasible (Burgers & Eckstein, 1980). As described earlier, creatine kinase was found to bind tightly to a 'transition-state analog complex' mixture comprised of creatine, MgADP and planar anions such as nitrate, nitrite and formate (Milner-White & Watts, 1971). Infrared spectroscopy showed that the anions were directly coordinated to the metal ion at the active site (Reed *et al.*, 1978), while EPR studies using Mn(II) and ^{17}O-labeling showed that the metal ion is bound to the α- and β-phosphates of ADP, to the anion, and to three water molecules (Reed & Leyh, 1980). A final EPR study suggested that an α,β,γ-tridentate complex of Mn(II) ATP is a substrate for the forward reaction and, in the product complex, Mn(II) remains coordinated to the α- and β-phosphates of MgADP as well as to phosphocreatine (Leyh *et al.*, 1985).

The CK-TSAC structure provided conclusive evidence that the earlier studies were indeed correct. In general, Mg(II) prefers a coordination number of six, and a preference for oxygen atoms as electron donating ligands (Katz *et al.*, 1996). In the TSAC structure the Mg^{2+} ion has octahedral coordination geometry involving three water molecules, two non-bridging oxygens from the α- and β-phosphates of ADP and an oxygen of the nitrate group. In the CK-MgADP structure, a water molecule replaces the nitrate ion (Lahiri *et al.*, 2002) while, in a subsequent structure with CK bound to MgAMPPNP, a non-hydrolyzable MgATP analogue, the Mg^{2+} ion interacts with three water molecules and the oxygens of the α,β,γ-phosphates of AMPPNP (Lahiri, 2004). The Mg^{2+} is liganded to the pro-*R* oxygen of the α-phosphate; thus not only did the early EPR studies correctly identify the coordination geometry of the Mg^{2+}, but the predictions of the stereochemical configuration of the metal nucleotide complex were also accurate (Reed & Leyh, 1980; Leyh *et al.*, 1982, 1985).

The Creatine Binding Site

Not surprisingly, the creatine binding site is much smaller than that of the nucleotide. The carboxylate of creatine forms a hydrogen bond to the main chain nitrogen of Val72 with the remainder of its interactions occurring via water molecules held in place by protein side-chains. Studies with alternative substrates had shown that replacement of the *N*-methyl group of creatine with the smaller hydrogen group (glycocyamine) or the bulkier ethyl group (*N*-ethylglycocyamine) led to a 43-or 11-fold reduction in binding affinity for the substrate, respectively (McLaughlin *et al.*, 1972). This specificity for creatine appears to be achieved by use of a binding "pocket" formed by Ile69 and Val325 (Figure 6B). These two hydrophobic residues are found on the two loops that close over the active site in the CK-TSAC structure and are not part of the active site in the CK-MgADP structure (Figure 6A). Mutagenesis studies with HMCK have subsequently revealed that

replacement of Ile69 by alanine results in greatly decreased enzymatic activity and complete loss of synergy (Novak *et al.*, 2004). Replacement of Ile69 by glycine in *Danio* MM-CK provided a similar result (Uda & Suzuki, 2004). In the same study, it was shown that replacement of Val75 with glycine led to an unstable enzyme, and the authors speculate that Val75 may be involved in stabilizing the flexible loop structure (Uda & Suzuki, 2004). In the C-terminal loop the substitution of Val325 in HMCK with glutamic acid results in a 100-fold preference for glycocyamine while replacement with alanine results in a slight preference for cyclocreatine (Novak *et al.*, 2004). Interestingly, in the AK structure Val325 is replaced by a negatively charged glutamic acid residue, Glu314, which interacts with the ε-nitrogen of the bound arginine. Sequence alignments had suggested that Asp326 was the CK homolog of Glu314, and replacement of Asp326 by alanine had resulted in a significant reduction in the value of k_{cat} for the forward reaction and an increase in the value of K_m for creatine (Cantwell *et al.*, 2001). Further, slight preference for cyclocreatine as a substrate was now apparent. At the time those results were discussed in terms of creatine binding but, when the CK-TSAC structure became available, it was clear that Asp326 in fact forms a salt bridge to His66 effectively "latching" the two loops into the closed position (Figure 7). The importance of His66 in catalysis by CK has also been demonstrated (Forstner *et al.*, 1997; Mourad-Terzian *et al.*, 2000), although it does not appear to be the acid-base catalyst identified in pH-rate profile studies (Cook *et al.*, 1981; Wang *et al.*, 2001). It is possible that the latch contributes to the electrostatic environment of the active site and assists in maintaining the conformation of the two loops so that the substrates are optimally aligned for catalysis. In any event, it is noteworthy that, from their respective positions in the MgADP structure, His66 and Asp326 move more than 25 Å to form the latch in the CK-TSAC structure. It had also been shown that at least one residue per monomer is labeled with diethyl pyrocarbonate, a reagent selective for histidine residues (Pradel & Kassab, 1968; Clarke & Price, 1979). The labeling occurred even if the thiol groups were reversibly blocked and there was no evidence for substrate protection by creatine (Pradel & Kassab, 1968). These results could be explained if His66 was the residue being labeled for, unless the loops are closed as in the CK-TSAC structure, His 66 is readily accessible to chemical modification agents and is located at some distance from the active site cysteine residue. That said, the studies of Chen *et al.* (1996) suggest that His296 is the likely target for the diethyl pyrocarbonate modification although the possibility that His66 is candidate was not addressed.

Studies with the conformationally restricted creatine analog, cyclocreatine, had indicated that the guanidino nitrogen cis to the methyl group was stereoselectively phosphorylated (Struve *et al.*, 1977; Phillips *et al.*, 1979). The CK-TSAC structure provides the structural basis for the stereospecificity. Not only does Glu232 form a bidentate salt bridge with the guanidino group, thereby stabilizing its positive charge, but it is also correctly positioned to act as a general base to remove a proton from the nucleophilic nitrogen. Glu232 is part of the NEED box that is conserved throughout the guanidino kinases and mutagenesis of this residue results in a severely impaired enzyme (Eder *et al.*, 2000b; Cantwell *et al.*, 2001). Even the most conservative replacement, by an aspartate, results in a 500-fold decrease in activity (Cantwell *et al.*, 2001). Similar results were obtained for arginine kinase, although the loss of activity was not as dramatic (Pruett *et al.*, 2003).

In addition to its interaction with Glu232, the guanidino group of creatine is also held in place through the interaction of its non-nucleophilic η1-nitrogen with Cys283. This is the 'reactive' cysteine described earlier, which is conserved throughout the guanidino kinases. Cys283 has a relatively low pK_a value of 5.4 suggesting that the optimal binding of creatine occurs when Cys283 is in the form of a thiolate anion (Wang *et al.*, 2001). Ser285 provides two interactions with Cys283, through its backbone carbonyl group and through its hydroxyl group, both of which contribute to its low pK_a value (Wang *et al.*, 2001; Naor & Jensen, 2004). In AK the serine is replaced by a threonine (Zhou *et al.*, 1998). Historically, one of the most contentious subjects for CK studies has been the importance of the reactive or as it was often termed, the "essential" cysteine residue. The controversy has arisen because of conflicting results from chemical modification studies with a variety of cysteine-specific reagents. In some instances the modified enzymes were completely inactive (Mahowald *et al.*, 1962; Zhou & Tsou, 1987; Wu *et al.*, 1989), whereas in others there was some residual activity (Smith & Kenyon, 1974; Der Terrossian & Kassab, 1976; Maggio *et al.*, 1977). Mutagenesis experiments have now demonstrated unequivocally that, while important, neither Cys283 nor Ser285 are essential for catalysis (Furter *et al.*, 1993; Wang *et al.*, 2001). Further, X-ray and kinetic

studies on mutants of the analogous cysteine residue in arginine kinase, also support this conclusion (Strong & Ellington, 1996; Gattis *et al.*, 2004). Taken together, the kinetic and mutagenesis data suggest that Cys283 is not indispensable, but it does help to keep creatine anchored and positioned for nucleophilic attack on the γ-phosphorus of MgATP (Lahiri *et al.*, 2002). In addition, it is possible that the basic character of the thiolate anion perturbs the resonance of the guanidinium by drawing positive charge toward the non-reactive $N_{\eta}1$, thereby increasing the nucleophilicity of the reactive nitrogen in the forward reaction and enhancing the leaving group properties of creatine in the reverse reaction (Gattis *et al.*, 2004).

The Nitrate Ion and Phosphoryl Group Transfer

Following the discovery of creatine kinase, it did not take long to recognize that the enzyme catalyzes the transfer of a phosphoryl, not a phosphate, group from ATP to creatine, and hence the early use of ATP-creatine transphosphorylase as a preferred name (Kuby & Noltmann, 1962). No evidence has been found for a phosphorylated enzyme intermediate, nor is there any exchange of phosphate between [^{32}P]ADP and ATP in the absence of guanidino substrates (Noda *et al.*, 1960). Equilibrium isotope exchange was used to demonstrate that the rate of phosphoryl transfer between ADP and ATP is similar to that between phosphocreatine and creatine (Morrison & Cleland, 1966), while quenched flow kinetics showed that phosphoryl group transfer is rate-limiting in both directions (Engelborghs *et al.*, 1975).

Potentially, the CK reaction involves a direct transfer of a phosphoryl group by an S_N2 type reaction, with the phosphoryl group forming an sp^3d hybrid in the transition state. It was proposed that the planar anions, such as nitrite and formate, mimicked the phosphoryl group in the transition state of the reaction (Milner-White & Watts, 1971). On that basis phosphoryl group transfer must occur via an 'in line' process as the planar anion is incompatible with an 'adjacent' transfer mechanism wherein the guanidine nitrogen of creatine forms part of the equatorial plane and the oxygen atoms of the phosphoryl group are not planar with the phosphorus atom (Milner-White & Watts, 1971). Although NMR data too, suggested that the phosphoryl group is transferred in a direct, in-line process (McLaughlin *et al.*, 1976), an S_N1 mechanism also would have a stable PO_3^- anion as a stable intermediate and, consequently, this mechanism could not be ruled out. Further evidence suggesting that the reaction proceeded via an associative (S_N2) rather than dissociative (S_N1) mechanism was found by Lowe and Sproat (1980) who determined that, in the absence of creatine or the presence of competitive inhibitors, there was no ^{18}O scrambling in adenosine 5′-[$\alpha\beta$-^{18}O, β-$^{18}O_2$]triphosphate. The fact that P_γ-OP_β bond cleavage followed by rotation around the P_β-OP_α bond and reformation of ATP would lead to scrambling, it was clear that the P_γ-OP_β bond must not be not broken in the absence of creatine. Subsequently, Hansen and Knowles (1981) used adenosine [γ-(*S*)-^{16}O, ^{17}O, ^{18}O]triphosphate to show that the reaction proceeds with inversion of configuration. Taken together, all the evidence is consistent with, but not definitive of, phosphoryl transfer occurring via an associative (S_N2) mechanism.

In the CK-TSAC structure, the nitrate anion is held in place by interactions with the Mg^{2+} ion, a water molecule which is also liganded to the Mg^{2+} ion, as well as with Arg236 and Arg320 which are located on either side of the anion. In general, divalent metal ions interact only weakly with anions, such as nitrate, and it is the interactions with the arginine residues that really stabilize this interaction. It is thought that, in the TSAC, the nitrate ion mimics the γ-phosphoryl group being transferred in the state intermediate between a dissociated metaphosphate and a pentavalent form (Reed & McLaughlin, 1973). In the CK-TSAC structure, the nitrate is not constrained by partial bonds to the β-phosphate and the guanidino nitrogen of creatine, as it would be for the γ-phosphate group in the true transition state. However, as shown in Figure 8, if a line is drawn which connects the guanidino $\eta 2$ nitrogen and the oxygen of the β-phosphate group, while passing through the center of the nitrate group, the plane of the anion is almost perpendicular to that line. The deviation

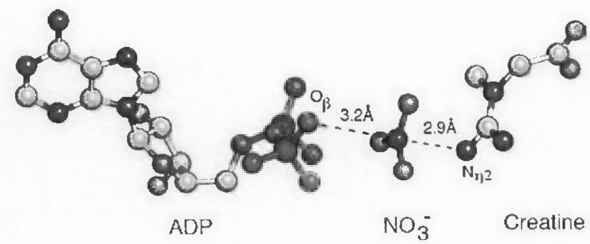


FIGURE 8 Ball and stick representation of the transition state complex formed by ADP, NO_3^- and creatine in the active site of TcCK. The nitrate group mimics the planar phosphoryl group during an in-line transfer between the ADP O_β and $N_{\eta}2$ of creatine.

from perpendicular, ~6°, is slightly greater than those observed for the analogous atoms in the refined AK-TSAC structure (Yousef *et al.*, 2002). Nonetheless, the distance between the guanidino $N_{\eta 2}$ and the ADP O_β, through the nitrate anion, is 6.1 Å, which is close to the 6 Å expected of the transition state for an associative in-line transfer mechanism (Yousef *et al.*, 2002).

Borders *et al.* (2003) noted that, in the AK-TSAC structure, the nitrate ion has an interaction with an asparagine residue as well as the arginine residues. The corresponding residue in the CK-TSAC structure, Asn285, is also positioned for a similar interaction (Lahiri *et al.*, 2002). Replacement of Asn285 in RMCK by aspartic acid led to a 15,000-fold reduction in k_{cat} for the mutant enzyme (Borders *et al.*, 2003). However, the values of K_m for both ATP and creatine were virtually unchanged, suggesting that Asn285 plays an important role in transition-state stabilization. The fact that no Asn285 mutant was able to form a TSAC lent further credence to that suggestion.

FUTURE DIRECTIONS

In general, this review has focused primarily on creatine kinase. Much of the forthcoming work on creatine kinase will need to be placed in context with studies on other guanidino (phosphagen) kinases. Consequently, in this brief summary, as yet unanswered questions about creatine kinase will also be considered in that framework.

One of the intriguing aspects of creatine kinase catalysis is the lack of any clearly indispensable residue. Glu232 appeared to be a prime candidate to act as a catalytic base but, while mutagenesis studies have shown that it is obviously important, the enzyme can get by without it (Brooks & Suelter, 1987; Eder *et al.*, 2000b; Cantwell *et al.*, 2001). The negatively charged NEED cluster is also conserved throughout the guanidino kinases and the X-ray structure of arginine kinase bound to a TSAC shows that the Glu232 homolog, Glu225, occupies the same position relative to the guanidino group (Zhou *et al.*, 1998). However, as with Glu232 in creatine kinase, mutagenesis of Glu225 results in reduced enzyme activity but the decrease is much less than may be expected for removal of a catalytic base (Pruett *et al.*, 2003). What is the precise role of Glu232, in particular, and the other residues in the NEED cluster?

For many years Cys283 was also thought to be essential although mutagenesis studies have now ruled that out (Furter *et al.*, 1993). As with Glu232 it is conserved throughout the guanidino kinases and has a homolog occupying the same position in the AK-TSAC structure (Zhou *et al.*, 1998), and mutants of the AK homolog also retain partial activity (Gattis *et al.*, 2004). Why does this cysteine residue have such a low pK_a? Is the presence of the thiolate anion mechanistically important for guanidino kinases?

Stroud has suggested that, for reactions involving two substrates, orientation of the substrates may be the most important element in catalysis. Further, the mechanism whereby substrates are aligned may be more important than any single catalytic residue (Stroud, 1996). Given the precise alignment of the components of the TSAC in both creatine (Lahiri *et al.*, 2002) and arginine (Yousef *et al.*, 2002) kinase, and the observation that even small deviations in the correct alignment drastically reduce arginine kinase activity (Pruett *et al.*, 2003), Stroud's comments are almost certainly appropriate for all the guanidino kinases. It is likely that both Glu232 and Cys283 play a significant role in the alignment of the substrates and that any involvement in acid-base catalysis is secondary. This, of course, has yet to be shown conclusively.

Although the recent structural data have answered many questions, they have raised many more. For example, precisely how do the guanidino kinases recognize their substrates? Using Table 1, it is possible to describe guanidino kinase substrates in relative terms as long (arginine, lombricine), short (creatine, glycocyamine), having an *N*-methyl group (creatine) and lacking an *N*-substituent (arginine, glycocyamine). As predicted by early sequence alignments (Suzuki *et al.*, 1997; Cantwell *et al.*, 2001), the comparison of open and closed structures of CK (Lahiri *et al.*, 2002) and AK (Yousef *et al.*, 2003) shows that much of the substrates specificity resides in the flexible loops. Sequence alignments would suggest that phosphorylation of long substrates is catalyzed by enzymes with short N-terminal loops, whereas that of short substrates is catalyzed exclusively by enzymes with long N-terminal loops (Suzuki *et al.*, 1997). Further, substrates with an *N*-methyl group such as creatine may require small hydrophobic residue (*e.g.*, Val325) on the C-terminal loop, while substrates lacking this *N*-substituent such as arginine and glycocyamine require an acidic group, *e.g.*, a glutamate, at the analogous position (Novak *et al.*, 2004). Unfortunately the initial results with AK/CK chimeras (Azzi *et al.*, 2004) are inconclusive as the lack of activity of

all the chimeras may be explained by the presence of Glu314 which interferes with the optimal positioning of creatine (Novak *et al.*, 2004). However, it is clear that such an approach will lead to a greater understanding of the specificity determinants of CK in particular, and the guanidino kinases in general.

The conzyme philosophy (Stroud, 1996) requires that the loops not only recognize the substrates, but help align the substrates for catalysis. A recent examination of the effects of mutations in the methyl specificity pocket in creatine kinase indicates that Ile69 in the N-terminal loop is important for synergism whereas Val325 in the C-terminal loop has a greater influence on substrate specificity (Novak *et al.*, 2004). In light of the conzyme proposal this is not surprising, but exactly what triggers the conformational changes necessary for correct substrate alignment and catalysis is a question that is yet to be addressed.

Other fundamental questions include why does CK prefer to be a dimer and AK a monomer? Can we make a stable and fully functional CK monomer? Is there crosstalk between the CK active sites? Of all enzymes, creatine kinase is one of the most intensively studied. However, despite more than 70 years of detailed investigation, a litany of questions remain to be answered.

ACKNOWLEDGEMENTS

We thank Mark Benson for assistance with the Figures, and both Malea Kneen and Pan-Fen Wang for careful reading of the manuscript. In addition we thank Ross Ellington for his helpful comments on the evolution of CK.

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REVIEW ARTICLE

Sports Med 2002; 32 (14): 903-944
0112-1642/02/0014-0903/$25.00/0

Oral Creatine Supplementation and Skeletal Muscle Metabolism in Physical Exercise[1]

José L.M. Mesa, Jonatan R. Ruiz, M. Marcela González-Gross, Ángel Gutiérrez Sáinz and *Manuel J. Castillo Garzón*

Department of Physiology, School of Medicine, University of Granada, Granada, Spain

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1 Dedicated to the memory of our admired Carlos Osorio Peláez (19.09.1932–27.06.2001), Professor of Physiology and Biochemistry at the School of Medicine of the University of Granada.

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Abstract

Creatine is the object of growing interest in the scientific literature. This is because of the widespread use of creatine by athletes, on the one hand, and to some promising results regarding its therapeutic potential in neuromuscular disease on the other. In fact, since the late 1900s, many studies have examined the effects of creatine supplementation on exercise performance. This article reviews the literature on creatine supplementation as an ergogenic aid, including some basic aspects relating to its metabolism, pharmacokinetics and side effects. The use of creatine supplements to increase muscle creatine content above ~20 mmol/kg dry muscle mass leads to improvements in high-intensity, intermittent high-intensity and even endurance exercise (mainly in nonweightbearing endurance activities). An effective supplementation scheme is a dosage of 20 g/day for 4–6 days, and 5 g/day thereafter. Based on recent pharmacokinetic data, new

regimens of creatine supplementation could be used. Although there are opinion statements suggesting that creatine supplementation may be implicated in carcinogenesis, data to prove this effect are lacking, and indeed, several studies showing anticarcinogenic effects of creatine and its analogues have been published. There is a shortage of scientific evidence concerning the adverse effects following creatine supplementation in healthy individuals even with long-term dosage. Therefore, creatine may be considered as a widespread, effective and safe ergogenic aid.

Creatine was discovered in 1835 by the French scientist Chevreul, and named after the Greek word *kreas* (flesh). The first creatine supplementation studies in animals and humans began in the early 1900s.[1-7] Since the discovery of phosphocreatine (PCr) in 1927 and the creatine kinase (CK) reaction in 1934, it is known that creatine is the substrate of CK to form PCr, a high-energy compound and an important energy store for ATP resynthesis in muscle.[8] Because of the widespread use of creatine by athletes,[9] promising results with regard to the clinical therapeutic potential of creatine in neuromuscular,[10-12] neurological[13-15] and cardiovascular[16,17] diseases, and the effects of creatine analogues as anticancer agents,[18,19] interest in creatine supplementation has grown exponentially over recent years, mainly with respect to using the compound as an ergogenic aid. It seems therefore opportune to review the ergogenic use of creatine. The aim of this article is to provide a comprehensive overview of the physiological and biochemical basis of the ergogenic use of creatine, including its metabolism, pharmacokinetics and side effects.

1. Biochemical and Physiological Aspects

This review will now provide an overview of the synthesis, transport and degradation processes of creatine in order to understand creatine metabolism in skeletal muscle.

1.1 Creatine Synthesis

Creatine or α-methylguanidinoacetic acid (figure 1) is a nitrogenous amino acid compound with a net positive charge and a molecular weight of 131Da. The endogenous synthesis of creatine (figure 2) involves three amino acids: glycine, arginine and methionine,[20] and begins with the transfer of

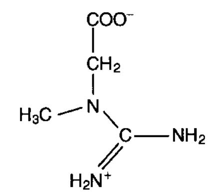

Fig. 1. Creatine (α-methylguanidinoacetic acid).

the amidino group of arginine to glycine to yield L-ornithine and guanidinoacetate. This reaction is catalysed by L-arginine:glycine amidinotransferase (AGAT). It has been theorised that guanidinoacetate is formed in the kidney and transferred via the blood to the liver.[8] In the hepatocyte, guanidinoacetate is methylated at the amidino group by the action of S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT). This reaction yields creatine and S-adenosyl-L-homocysteine. The rate-limiting step in creatine synthesis is the formation of guanidinoacetate by AGAT.[21] Creatine is capable of inducing feedback inhibition of AGAT possibly by inhibiting some steps before translation of AGAT mRNA.[8,21] The endogenous synthesis of creatine is 1–2 g/d,[21-23] and occurs mainly in the liver[24] and secondarily in the pancreas and kidney.[21] An additional 1–2 g/d of creatine are obtained from dietary intake, mainly fish and meat (4.5g creatine/kg salmon and beef).[21,25] Endogenous creatine synthesis is downregulated by diet and, therefore, reduced after enhanced creatine ingestion,[21,26,27] but normal secretion rates return upon termination of supplementation.[28]

1.2 Creatine Transport into Muscle Cells

Muscle fibres are unable to synthesise creatine, therefore, it must be taken up from the blood-

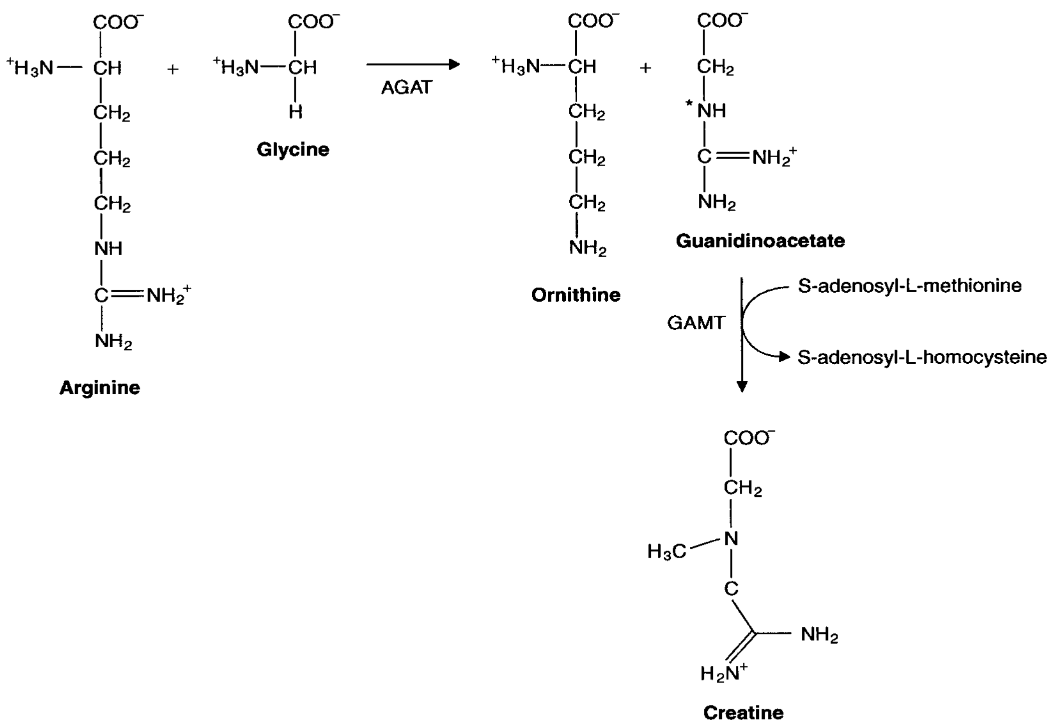

Fig. 2. Endogenous synthesis of creatine. In the kidney, arginine and glycine yield L-ornithine and guanidinoacetic acid by L-arginine: glycine amidinotransferase (AGAT). In the hepatocyte, a methyl group from S-adenosyl-L-methionine is transferred to the nitrogen atom (marked by asterisk) by S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT), yielding creatine and S-adenosyl-L-homocysteine.

stream. The daily demand for creatine is met both by intestinal absorption of dietary creatine and by the novo creatine biosynthesis. Creatine is, therefore, exported from both liver and gut, and accumulated in CK-containing tissues. In healthy omnivorous individuals, normal plasma creatine levels range from 50–100 μmol/L.[29,30] These figures decrease to 25–32 μmol/L in vegetarians because of their lower exogenous intake.[31] Over 90% of creatine enters skeletal muscle by binding to a specific transporter protein.[23] This saturable and Na^+-K^+ ATPase mediated[22,32] mechanism depends of extracellular [Na^+] and [Cl^-][33,34] (where brackets denote concentration) and allows creatine to enter the muscle against a concentration gradient.[32,35] This transport mechanism shows an apparent Michaelis-Menten constant (K_m) for creatine in the micromolar range (15–30 μmol/L),[35] so the normal plasma ranges are sufficient for adequate creatine transport in muscle.

1.2.1 Creatine Transporters in Muscle

Two specific transporters, present in muscle fibre membranes, take up creatine from the bloodstream. These are creatine transporter 1 (CRT1) and choline transporter 1 (CHOt1).

Creatine Transporter 1

CRT1 is expressed in cardiac and skeletal muscle, together with brain, kidney and placenta.[35] CRT1 presents twelve transmembrane domains and shows two potential N-glycosylation sites.[35,36] This creatine transporter belongs to the superfamily of Na^+ and Cl^- dependent neurotransmitter transporters.[37] The stoichiometry of creatine, Na^+ and Cl^- seems to be 1 : 2 : 1.[34] Although the specific structure/function relationship of the CRT1 has not been studied,[36] Sora et al.[35] dem-

onstrated greatest identity with γ-aminobutyric acid (GABA) and taurine transporters. To date, the cDNAs for human, rat, rabbit and torpedo fish CRT1 creatine transporters have been identified.[33,35,38-40] The human CRT1 gene is located on chromosome Xq28, it contains 13 exons and spans approximately 8.5kb of genomic DNA.[40,41] In rat muscle, the amount CRT1 present in type I fibres is higher than in type II fibres.[42] It is probable in these animals, but not in humans, that type I muscle fibres take up more creatine than type II fibres. Low concentrations of CRT1 induce low concentrations of creatine in muscle fibres, as occurs in human myopathies.[43]

Choline Transporter 1

A rabbit homologue of the putative rat choline transporter CHOT1 has been shown to mediate creatine transport.[33] Northern blot analysis revealed a major transcript of 4.8kb in brain, heart, skeletal muscle and kidney, showing CHOT1 as a widely expressed rat creatine transporter.[44]

1.3 Creatine Degradation

In muscle cells at rest, creatine is phosphorylated by CK to form PCr within 25 minutes upon arrival (figure 3). For this purpose, the ATP formed by glycolysis and oxidative phosphorylation reacts with creatine to form ADP and PCr. Large negative charges on PCr prevent diffusion across biological membranes thus locking PCr in the muscle cell.[45] During exercise, when muscle ATP is being consumed, the high-energy phosphoryl group of PCr is transferred to ADP to restore ATP. Creatine is then recycled or transformed to creatinine (Crn). Crn cannot be reutilised and is excreted in the urine. The dietary

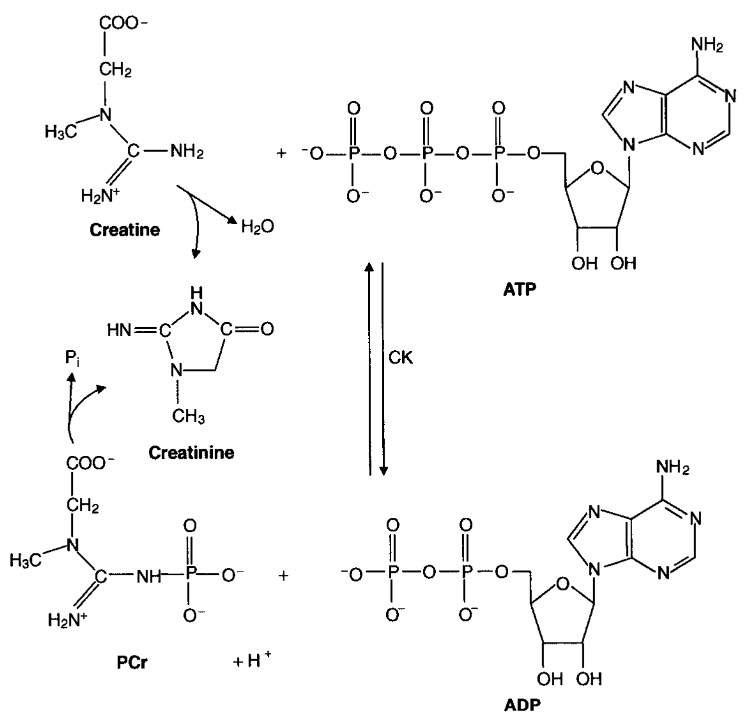

Fig. 3. Creatine kinase (CK) reaction. Creatine is the substrate of CK to form phosphocreatine (PCr). All CK isoenzymes catalyse the reversible transfer of the γ-phosphate group of ATP to the guanidino group of creatine to yield ADP, PCr and H^+. PCr (at a rate of 2.6%) and creatine (at a rate of 1–2%) are degraded daily to creatinine by spontaneous non-enzymatic reactions. **P_i** = inorganic phosphate.

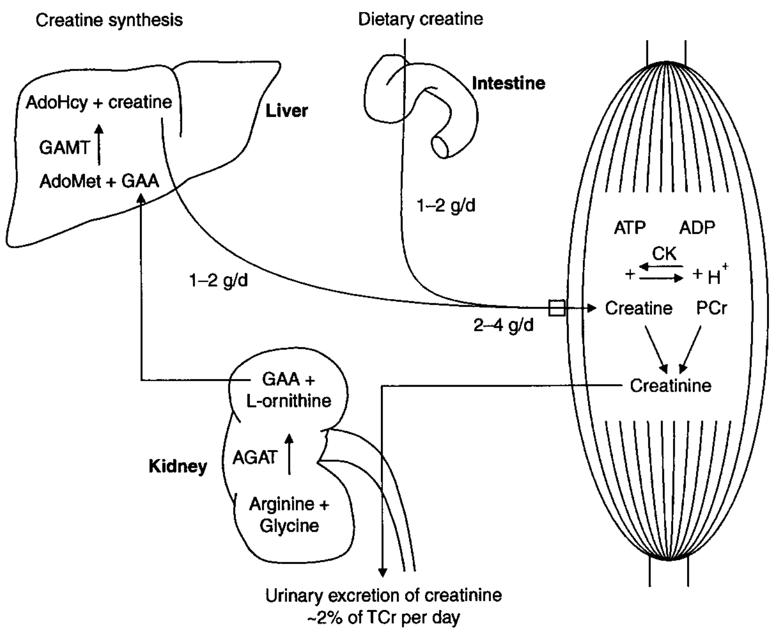

Fig. 4. General creatine metabolism in the human body. The daily demand for creatine is met both by intestinal absorption of dietary creatine (1–2g) and by *de novo* creatine biosynthesis (1–2g). The first step of creatine biosynthesis probably occurs mainly in the kidney, whereas the liver is likely to be the principal organ accomplishing the subsequent methylation of guanidinoacetic acid (GAA) to creatine. Most (~95%) of the creatine is found in muscle cells. Because muscle has virtually no creatine-synthesising capacity, creatine has to be taken up from the blood against a large concentration gradient by a saturable [Na^+] and [Cl^-] (where brackets denote concentration) dependent creatine transporter that spans the plasma membrane. The muscular creatine and phosphocreatine (PCr) are nonenzymatically converted at an almost steady rate (~2% of total creatine [TCr = PCr + creatine] per day) to creatinine, which diffuses out of the cells and is excreted by the kidneys into the urine. **AdoHcy** = S-adenosyl-L-homocysteine; **AdoMet** = S-adenosyl-L-methionine; **AGAT** = L-arginine:glycine amidinotransferase; **CK** = creatine kinase; **GAMT** = N-guanidinoacetate methyltransferase.

intake and endogenous production of creatine matches the spontaneous degradation of PCr and creatine to Crn at a rate of 2.6% and 1–2% per day, respectively.[21,26,46,47] Therefore, Crn production totals 2 g/d based on a 70kg human and a total creatine pool of 120g.[21] The Crn turnover is proportional to the muscle mass and increases with high-intensity physical exercise.[48] Once Crn is formed, it enters the circulation by diffusion and is eliminated from the body through glomerular filtration.[49] Vegetarians have marginally lower urinary Crn excretion rates than individuals on normal diets, suggesting that creatine biosynthesis rates and muscle creatine contents are also marginally lower than in individuals ingesting creatine-containing diets.[31]

In summary, the daily demand for creatine is met both by intestinal absorption of dietary creatine (1–2g) and by *de novo* creatine biosynthesis (1–2g) [figure 4]. Because muscle has virtually no creatine-synthesising capacity, creatine has to be taken up from the blood against a large concentration gradient by a saturable [Na^+] and [Cl^-] dependent creatine transporter that spans the plasma membrane. Muscular creatine and PCr are nonenzymatically converted at an almost steady rate (~2% of total creatine per day) to Crn, which diffuses out of the cells and is excreted by the kidneys into the urine.

1.4 Creatine Distribution in the Body

Total body creatine (TCr) includes both creatine and PCr. Approximately 95% of the TCr is found

in skeletal muscle.[21] High creatine levels are also found in the heart, spermatozoa and photoreceptor cells of the retina. Intermediate levels are found in the brain, brown adipose tissue, intestine, seminal vesicles, seminal vesicle fluid, endothelial cells, and macrophages; and only low levels are found in the lung, spleen, kidney, liver, white adipose tissue, blood cells and serum.[22,50-56] In 1974, Harris et al.[57] reported a mean muscle TCr content in humans of 124.4 (range: 110–160) mmol/kg dry mass (dm), of which 49.0 mmol/kg dm corresponded to creatine and 75.5 mmol/kg dm to PCr. However, it can vary widely among individuals (~90–180 mmol/kg dm),[58] with ~60% of it in the form of PCr and ~40% in the form of creatine.[25,59-61] The ratio of PCr to TCr (PCr/TCr) is indicative of the PCr energy charge, and this ratio normally ranges from 0.6 to 0.8 in resting skeletal muscle.[60] TCr content in resting muscle tissues in humans is influenced by several factors, which will be discussed in sections 1.4.1–1.4.4.

1.4.1 Influence of Muscle Fibre Type

Creatine and PCr concentrations correlate with the glycolytic capacity of the different skeletal muscles.[62] In this regard, the resting PCr content is 5–30% higher in type II versus type I muscle fibres[63-66] (figure 5). The previous findings were confirmed by a nuclear magnetic resonance (NMR) study that investigated the relationship between PCr content and muscle fibre type composition in human skeletal muscle.[67] This study reported a significantly higher PCr content in type II versus type I muscle fibres. This is in agreement with the higher levels of muscle PCr observed in sprinters, whose muscles also contain a higher proportion of type II fibres.[68,69] In addition, it has been reported that type I fibres have a lower creatine content than type II fibres.[70] In the same study, within type II fibres resting PCr increased with increasing myosin heavy chain IIX isoform content (r = 0.59, p < 0.01). The content of TCr is therefore dependent on the skeletal muscle fibre type.

1.4.2 Influence of Age

Using muscular biopsies, Moller et al.[71] reported that PCr concentrations were approximately 5% lower in the elderly (52–79 years) compared with men and women about 40 years younger. This finding has been confirmed by more recent NMR[72-74] and muscle biopsy[75,76] studies. Therefore, muscle PCr content decreases as a function of age. This can be attributed, at least in part, to the lower content of type II muscle fibres present in elderly individuals.[75] Nevertheless, the previous studies were performed in healthy but untrained individuals. Consequently, it is difficult to determine whether the decrease in muscle PCr is caused by aging itself or is simply caused by inactivity. Further research is necessary in order to clarify this topic.

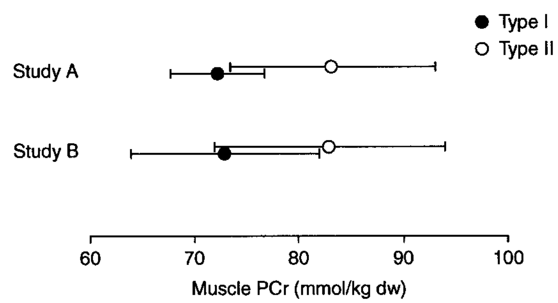

Fig. 5. Resting muscle phosphocreatine (PCr) content in both type I and II muscle fibres in humans. Resting PCr content is higher in type II *vs* type I muscle fibres. Values are expressed as means and SDs (error bars). **Study A** = Söderlund and Hultman;[63] **Study B** = Tesch et al.;[66] **dw** = dry weight.

1.4.3 Influence of Gender

Comparisons of creatine and PCr levels in skeletal muscle in males and females appear equivocal, with one study reporting higher total creatine levels relative to tissue weight in women,[77] while other studies reported no significant differences between males and females.[76,78] By contrast, TCr is clearly increased in the muscle tissue of pregnant women.[79] There is, therefore, limited evidence that TCr levels are lower in women than men, especially when accounting for muscle mass.

1.4.4 Influence of Training Status

A study using NMR spectroscopy failed to show any significant difference between trained and untrained individuals in muscle PCr content.[80] Other studies[81-83] reported higher levels of PCr in the quadriceps muscles of sprinters compared with long-distance runners. However, since

sprinters have more type II muscle fibres than long-distance runners, and the resting PCr content is higher in type II than in type I muscle fibres,[63-66] these differences may be mainly caused by fibre type and not training effects. In any case, there is not, at present, enough evidence to suggest that differences exist between trained and untrained individuals (both men and women) with regard to TCr content. Further research is therefore required on this topic.

1.5 Functions of Creatine in Muscle Cells

There are five general functional aspects related to the creatine/PCr system in muscle cells. These are outlined below.

1.5.1 Buffering ATP and ADP Content

During a short period of intense physical exercise, muscle ATP content may be partially buffered and restored after muscle activity has ceased. During physical inactivity, muscle ADP content is buffered. In fact, the formation of CK-mediated PCr allows the conversion of ATP to ADP, maintaining the substrate for new phosphorylation reactions.

Buffering Muscle ATP Content During Physical Exercise

A large pool of PCr is available in type II fibres for immediate regeneration of ATP hydrolysed during short periods of intense work (see section 1.4.1). This PCr hydrolysis buffers, at least in part, muscle ATP content during physical exercise in both type I and type II muscle fibres (figure 6). Nevertheless, after 10–30 seconds of maximal exercise, the PCr hydrolysis mediated diminution of muscle PCr is higher in type II than in type I muscle fibres.[66,84,85] Since ATP turnover rates occur in muscle up to 10–15 mmol/kg/sec and the PCr content is limited (~70–90 mmol/kg dm), the relative importance of PCr hydrolysis as a source of ATP regeneration falls off dramatically as the exercise duration lasts beyond a few seconds.[86] In fact, during a 6-second sprint, at a power output representing 250% maximal oxygen uptake ($\dot{V}O_{2max}$), PCr hydrolysis and glycolysis each contribute 50% to the total ATP requirement with very little contribution from oxidative phosphorylation.[87] However, during a 30-second sprint averaging ~200% $\dot{V}O_{2max}$, PCr hydrolysis only contributes ~25% to the ATP requirements[88] (figure 7).

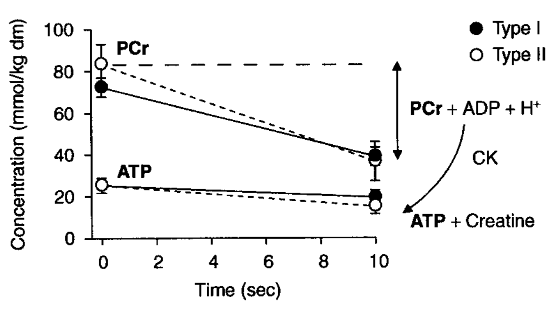

Fig. 6. Kinetics of muscle ATP and phosphocreatine (PCr) in both type I and type II muscle fibres during a 10-second all-out exercise bout. ATP is buffered in part during physical exercise by PCr hydrolysis. Therefore, PCr decreases during physical exercise in a higher amount than ATP in both type I and type II muscle fibres. Values are expressed as means and SDs (error bars).[63,66,85] **CK** = creatine kinase; **dm** = dry mass.

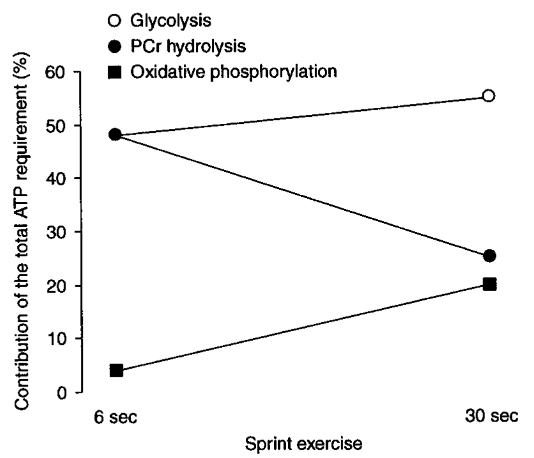

Fig. 7. Contribution of the total ATP requirement in both 6- and 30-second sprint exercises. During a 6-second sprint at a power output representing ~250% maximal oxygen uptake ($\dot{V}O_{2max}$), phosphocreatine (PCr) hydrolysis contributes ~50% of the total ATP requirement, whereas during a 30-second sprint averaging ~200% $\dot{V}O_{2max}$, PCr hydrolysis only contributes ~25%.[87,88]

Restoring Muscle ATP Content After Physical Exercise

Muscle ATP has to be restored to resting values after physical exercise; this is partially accomplished through PCr hydrolysis, which also has to

be resynthesised. In the past, a simple monoexponential model was proposed to describe the time course of PCr concentration change after exercise.[89,90] However, more recent studies[91-93] found PCr to recover initially more rapidly and thereafter more slowly than a simple monoexponential model would indicate. In addition, Söderlund and Hultman[63] found that, during recovery, PCr concentration in type II muscle fibres was higher than in resting muscle before exercise. Similar findings were obtained in cat soleus muscle by Kushmerick et al.[94] as well as in human anterior tibialis muscle[95] (figure 8). Since it is impossible for the monoexponential curve to over- or undershoot the level observed at rest, Nevill et al.[95] proposed a double-exponential model for PCr resynthesis. In the study of Söderlund and Hultman[63] and in the Nevill et al.[95] study, the exercising muscle was occluded, and recovery was initiated by a sudden return of blood to the leg. Because the supply of oxygen to tissue and the removal of metabolites such as lactate and H^+ determine the rate of PCr resynthesis,[96] this higher blood flow to the muscle tissue may improve the PCr resynthesis during recovery, explaining the overshoot observed in these studies. After physical exertion this PCr resynthesis allows ATP resynthesis in muscle cells by CK reaction after physical exertion. PCr resynthesis after physical exercise is an aerobic process,[97] being proportional to the mitochondrial oxygen uptake ($\dot{V}O_2$) rate.[90,98-103] In this regard, PCr resynthesis in type I is higher than in type II muscle fibres after 60 seconds of recovery.[66] Finally, PCr resynthesis is lower in elderly than in young individuals,[72,101,104-106] decreasing 8% every 10 years in individuals over 30 years of age.[106]

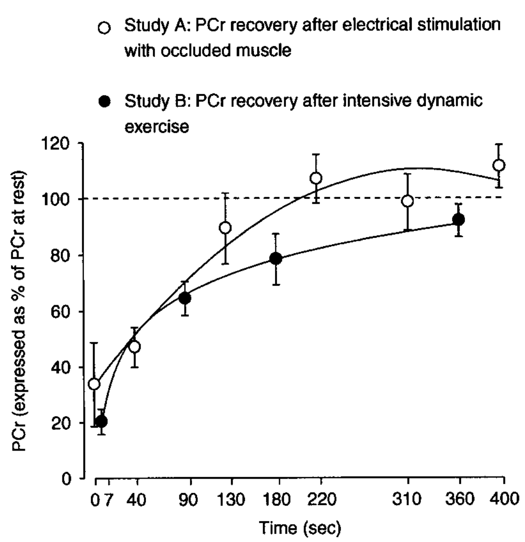

Fig. 8. Phosphocreatine (PCr) recovery after maximal exercise. Data described here are taken from two studies; study A used ^{31}P-magnetic resonance spectroscopy after maximal electrical stimulation of the anterior tibialis with occluded muscle; study B used needle biopsy tissue from the vastus lateralis after intensive dynamic exercise. A transient overshoot is observed in PCr recovery after physical exertion with occluded muscle. This overshoot may be caused by a sudden return of blood to the leg after occluded muscle, removing more H^+ and, therefore, varying the rate of PCr resynthesis during recovery. Values are expressed as means and SDs (error bars) [reprinted from Nevill et al.,[95] with permission from the publisher].

Buffering Muscle ADP Content

In periods of muscle inactivity, less ATP is needed by muscle cells. In these situations CK catalyses the reversible transfer of the γ-phosphate group of ATP to the guanidino group of creatine to yield ADP, PCr and H^+. Therefore, the formation of PCr allows the conversion of ATP to ADP, maintaining the substrate for new phosphorylation reactions. Because of the high cytosolic CK activity in these muscles, the CK reaction remains in a near-equilibrium state, keeps muscle ADP and ATP contents almost constant (over several seconds), and thus 'buffers' the cytosolic phosphorylation potential that seems to be crucial for the adequate functioning of a variety of cellular ATPases.[8]

1.5.2 'Shuttle' Hypothesis for the Creatine Kinase System

Two isoforms of CK exist in mitochondria: ubiquitous mitochondrial CK (Mi-CK) and sarcomeric Mi-CK. Both are located in the mitochondrial intermembrane space and both form homodimeric and homo-octameric molecules that are readily interconvertible. Distinct CK isoenzymes are associated with different sites. Mi-CK is in the mitochondrial intermembrane space (site of ATP production), and cytosolic CK is bound to the myofibrillar M line, the sarcoplasmic reticulum, or the

plasma membrane (sites of ATP consumption). It is well known that type I muscle fibres and myocardial fibres depend on a more continuous delivery of high-energy phosphates to the sites of ATP utilisation. According to the 'transport' ('shuttle') hypothesis for the CK system,[8,107-115] the γ-phosphate group of ATP, synthesised within the mitochondrial matrix, is transferred by Mi-CK in the mitochondrial intermembrane space to creatine to yield PCr, ADP and H^+. ADP liberated by the Mi-CK reaction may be directly transported back to the matrix where it is rephosphorylated to ATP. PCr leaves the mitochondria and diffuses through the cytosol to the sites of ATP consumption. CK isoenzymes of these sites regenerate ATP and thus warrant a high phosphorylation potential in the intimate vicinity of the respective ATPases. Liberated creatine, diffuses back to the mitochondria, closing the cycle (figure 9). According to this hypothesis, transport of high-energy phosphates between sites of ATP production and ATP consumption is achieved mainly (but not exclusively) by PCr and creatine. For the buffer function, no Mi-CK is required. Mi-CK may be a prerequisite for efficient transport of high-energy phosphates, especially if diffusion of adenine nucleotides across the outer mitochondrial membrane is limited. In accordance with these ideas, the proportion of Mi-CK seems to correlate with the oxidative capacity of striated muscles.[8]

1.5.3 Buffering of pH

Because net PCr hydrolysis consumes hydrogen ions, it may contribute to buffering intracellular acidosis during physical exercise when the CK reaction favours ATP resynthesis from PCr.

1.5.4 Regulation of Glucose and Glycogen Metabolism

PCr partly inhibits phosphofructokinase (PFK), a key glycolytic enzyme and directional flow valve. During intense physical exercise, PFK becomes less inhibited because PCr decreases. Therefore, during intense physical exertion, the rate of glycolysis increases. In addition, when humans and animals are depleted of tissue creatine, they adapt by increasing oxidative enzymes such as Mi-CK, succinate dehydrogenase, citrate syn-

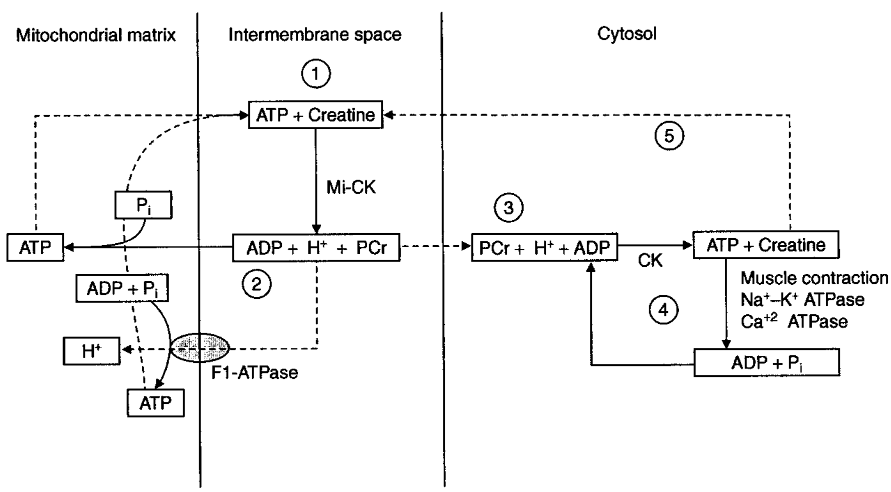

Fig. 9. 'Shuttle' hypothesis for the creatine kinase (CK) system. **(1)** ATP synthesised within the mitochondrial matrix is used by mitochondrial CK (Mi-CK) to phosphorylate creatine in the intermembrane mitochondrial space, yielding phosphocreatine (PCr), ADP and H^+; **(2)** the ADP liberated by this reaction may be transported back to the matrix where it is rephosphorylated to ATP, in addition, the liberated H^+ may yield ATP by F_1-ATPase; **(3)** PCr leaves the mitochondria and diffuses through the cytosol to the sites of ATP consumption (myofibrillar M line, sarcoplasmic reticulum or plasma membrane), where **(4)** CK isoenzymes regenerate ATP, which is used as a substrate by the respective ATPases. The ADP liberated in these reactions is used as a substrate for the cytosolic CK reaction; **(5)** the creatine liberated by the CK reaction diffuses back to the mitochondria, closing the cycle. P_i = inorganic phosphate.

thase, and GLUT-4 glucose transporters.[116,117] All of these proteins are involved in aerobic metabolism and can offset the lack of anaerobic energy supplied by the PCr system.

1.5.5 Membrane Stabilisation

PCr may stabilise the plasma membrane because of the zwitterion nature of the PCr molecule with its negatively charged phosphate and acidic groups and its positively charged guanidino groups (figure 3). PCr binds to the phospholipid head groups and thus decreases membrane fluidity and decreases the loss of cytoplasmic contents.[28]

2. Pharmacokinetics

To date, much of the work on creatine has focussed on its use as an ergogenic aid and on its clinical effects rather than on characterising the pharmacokinetics, thus leaving a research gap. It is difficult to compare studies of creatine pharmacokinetics because of differences in the study design, creatine products, and methods of analysis. In addition, many of the reported studies are incomplete with regard to pharmacokinetic analysis, and further research is necessary in order to establish standard pharmacokinetic parameters.

2.1 Pharmacokinetics of a Single Dose

2.1.1 Absorption

After creatine ingestion, creatine can pass through the gastrointestinal tract epithelia into the blood. Oral absorption of creatine depends on the physicochemical properties of the molecule and on the splanchnic blood flow. If one considers the latter factor as being constant, and bearing in mind that creatine may be administered orally either as a solution or solid, is the form of creatine ingestion the main factor responsible for alterations in creatine absorption rate? In this regard, Harris et al.[118] examined the plasma concentration curve obtained over 6 hours after ingestion of 2g of creatine either in solid form or in solution. Creatine ingested as a solid form (as a lozenge or as a crystalline suspension in ice-cold water) resulted in a 20% lower peak concentration compared with the same dose administered in solution. The authors concluded that creatine administered in the form of meat or as other solid forms is readily absorbed but may result in slightly lower peak concentrations than when the same dose is ingested as a solution.

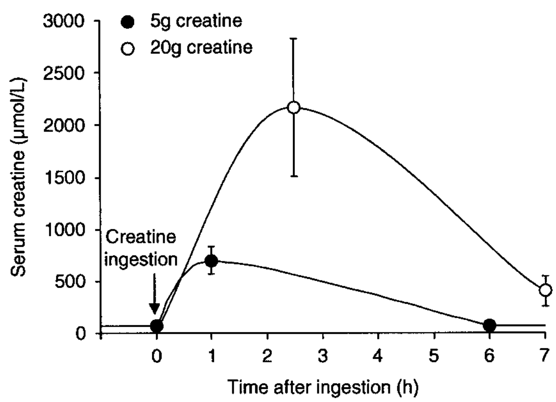

Fig. 10. Serum creatine kinetics after oral creatine ingestion. Fasting serum values of creatine are about 50–100 μmol/L in humans. Following 5g creatine ingestion, the peak value of serum creatine occurs in 1 hour, reaching plasma values of 600–800 μmol/L. The return to fasting values occurs after 5–7 hours. In response to 20g creatine, plasma creatine concentration increases by 50-fold (peak value of 2170 ± 660 μmol/L), and this occurs approximately 2.5 hours after ingestion. Values are means and SDs (error bars).[30,119,120]

2.1.2 Peak Values in Blood

Following 5g of oral creatine solution, the peak value of serum creatine occurs in 1 hour, reaching plasma values of 600–800 μmol/L.[30,119] In response to lower doses (<2g creatine), the blood increase in creatine is insignificant. By contrast, in response to higher doses (20g creatine), plasma creatine concentration increased by 50-fold (peak value of 2.17 ± 0.66 mmol/L), and this occurred approximately 2.5 hours after ingestion[120] (figure 10). In this respect, we may consider that oral administration of medium-low doses of creatine (around 5g) in humans demonstrates a time to maximal plasma concentration (T_{max}) of less than 2 hours, whereas at doses above 10g, T_{max} increases to over 3 hours.[28]

2.1.3 Clearance

After 5g of oral creatine ingestion, the return to fasting values (50–100 μmol/L) occurs after 5–7 hours.[30,119] Creatine can be cleared from the blood via two parallel pathways. The first is a saturable uptake (see section 1.2) into various organs

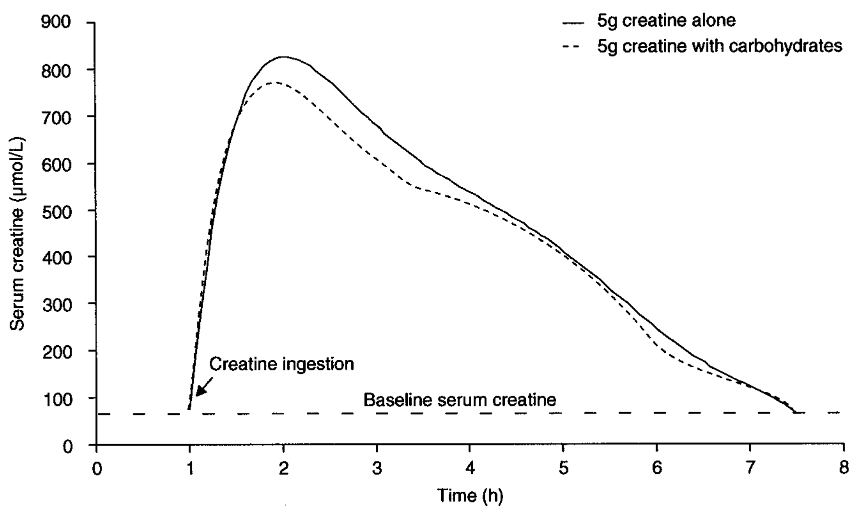

Fig. 11. Usefulness of the area under the plasma creatine concentration-time curve (AUC) after creatine ingestion. In both theoretical curves 5g creatine were ingested, with or without concomitant intake of carbohydrates. To determine AUCs of plasma creatine, equations of both curves are necessary. Then the area is calculated by integral calculation. The theoretical curve of 5g creatine alone was defined by equation 1 $y_1 = -0.8118x^6 + 23.756x^5 - 279.42x^4 + 1683.9x^3 - 5459.4x^2 + 8848.1x - 4741.8$, whereas the curve of 5g creatine with carbohydrates was defined by equation 2 $y_2 = -1.2416x^6 + 35.013x^5 - 394.47x^4 + 2260.1x^3 - 6907.6x^2 + 10506x - 5420.9$. For both equations, y is the serum creatine content (μmol/L), and x is the time (h). Then y_1 AUC is calculated by defined integral [1, 7.5] of y_1 = 3040.60 μmol/L • h, and y_2 AUC is calculated by defined integral [1, 7.5] of y_2 = 2836.06 μmol/L • h. For 6.5 hours (7.5 –1), y_2 AUC (5g creatine with carbohydrates) is less than y_1 AUC (5g creatine alone). Since the amount of creatine ingestion is the same in both cases, this decrement may be caused by both a major creatine uptake by muscle cells and/or a higher creatine excretion into the urine. Considering a constant urinary excretion of creatine for a 6.5-hour period (7.5 –1) after 5g creatine ingestion with carbohydrates, 3040.60 –2836.06 = 204.54 μmol/L • h more than 5g creatine-alone ingestion may be taken by muscle cells.

and cells (mainly muscle tissue). It will be mentioned later (see section 2.3) that several factors affect this uptake. The second pathway is renal elimination. This second pathway is used when creatine uptake by cells is saturated, so it is probably not used after a single dose of 5g creatine.

2.1.4 Usefulness of the Area Under the Plasma Creatine Concentration-Time Curve After a Single Dose

Since creatine in the blood may only be taken up by tissues (mainly muscle) and eliminated by the kidney (as creatine and Crn), the area under the plasma creatine concentration-time curve (AUC) coupled with urinary creatine and Crn excretion is a marker of creatine uptake by muscle. For example, a lower AUC of plasma creatine when creatine is ingested with carbohydrates compared with when it is ingested alone, would indicate higher creatine uptake by muscle when creatine is ingested with carbohydrates (figure 11).

2.1.5 Intravenous Administration

Intravenous administration of creatine is used in heart surgery, as well as by elite sportsmen to improve creatine uptake by muscle cells. In Italy, creatine is manufactured at 0.5, 1 and 5g doses. The appropriate intravenous dose to raise the plasma creatine concentration above 400 μmol/L (optimal for muscle uptake) should range from 300–600mg creatine. The optimal intravenous injection of creatine, its safety, and its half-life in plasma, remain to be elucidated. In fact, to our knowledge, the half-life of plasma creatine after intravenous injection has been studied only for small amounts (0.1–3mg creatine), and ranges from 20–70 minutes.[121]

2.2 Pharmacokinetics of Multiple Dosages

When creatine is used as an ergogenic aid, it is usually administered over several days. It is therefore necessary to analyse its pharmacokinetics during multiple dosages.

2.2.1 Creatine Retention by Tissues

When creatine is administered at a rate of 20 g/d over several days, almost 30% of the administered creatine is retained during the initial 2 days of supplementation, but this percentage decreases to only 15% from days 2 to 4.[4,122] Thus, most of the ingested creatine is retained by the body (mainly muscle tissue) in the first few days of administration, and most ingested creatine (85–90%) is excreted as creatine and Crn in the urine with continued daily supplementation. Green et al.[119] investigated the effects of creatine ingestion on plasma creatine levels at days 1 and 3 of a 2-day, 20 g/d regimen. Following a 5g dose on day 1, plasma creatine reached a peak value of 170 mg/L at a T_{max} of 50 minutes. On day 3, after a 5g dose, plasma creatine had a peak value of 234 mg/L at the same T_{max}. Interestingly, the authors found a lower AUC (about 7%) on day 1 than on day 3. This difference was probably caused by incomplete clearance of creatine from the blood on day 3, since on day 1 plasma creatine reached near baseline levels by 270 minutes, whereas on day 3 plasma creatine was 7 times higher than baseline values at 270 minutes. These data suggest a reduced muscle uptake after 2 days of 20 g/d oral creatine administration (figure 12). Taken together, these data suggest an impairment of exogenous creatine retention into cells with creatine administration of 20 g/d for more than 2 days. Following these results and others (see section 2.2.2), we proposed a schema of creatine supplementation (see section 2.4), but more studies are needed to clarify the pharmacokinetic effects of multiple doses of creatine and its changes in relation to age, training status, gender and previous TCr content in muscle.

2.2.2 Creatine and Creatinine Excretion During Multiple Dosages

As previously discussed (see section 2.1.4), elevated plasma creatine levels after multiple creatine doses (over several days) are caused by lower tissue uptake. In fact, Kamber et al.[123] reported that after creatine administration (20 g/d, for 5 days) basal plasma creatine increased from 62 ± 5 to 155 ± 29 μmol/L, due to saturated creatine consumption by tissues. The creatine excess may be

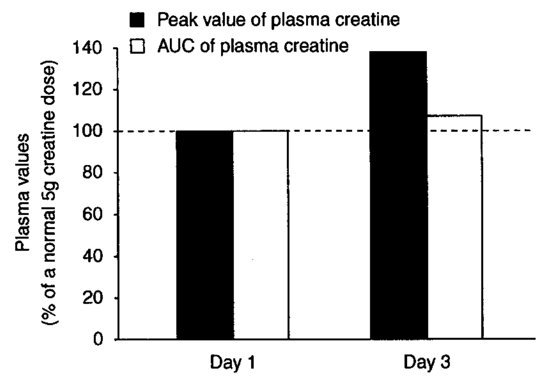

Fig. 12. Creatine pharmacokinetics at day 1 and day 3 of a 2-day, 20 g/d regimen. Both peak values of plasma creatine and the area under the plasma creatine concentration-time curve after creatine ingestion (AUC) of plasma creatine after a single 5g creatine dose are higher at day 3. Considering constant urinary excretion of plasma creatine, these results suggest less creatine uptake by tissues (mainly muscle tissue) at day 3. Values are expressed as percentage of plasma values after a normal 5g creatine dose.[119] Dotted horizontal line represents AUC of plasma creatine after a 5g creatine dose administered on day 1 (100%).

excreted in urine; however, the creatine spillover by urine is minimised because it has been suggested that CRT1 is also found in the kidney and may serve to reabsorb creatine from the urine.[8] Thus, creatine recirculated in plasma may be degraded non-enzymatically to Crn. Unfortunately, results are controversial concerning plasma Crn after creatine supplementation.[123-130] This means that one or more unknown covariables influence plasma Crn after creatine administration. Since these were randomised placebo-controlled studies, the covariable is probably the kind of creatine administration. Accordingly, we classified these studies into two groups: (i) carbohydrate group, where creatine was administered in conjunction with carbohydrates; and (ii) noncarbohydrate group, where creatine was administered alone (figure 13). This figure shows that plasma Crn is lower when creatine is administered with carbohydrates. As will be discussed later (see section 2.3.1), creatine uptake by tissues can be substantially augmented when creatine is ingested in conjunction with large quantities of simple carbohydrates able to stimulate endogenous insulin secretion. There-

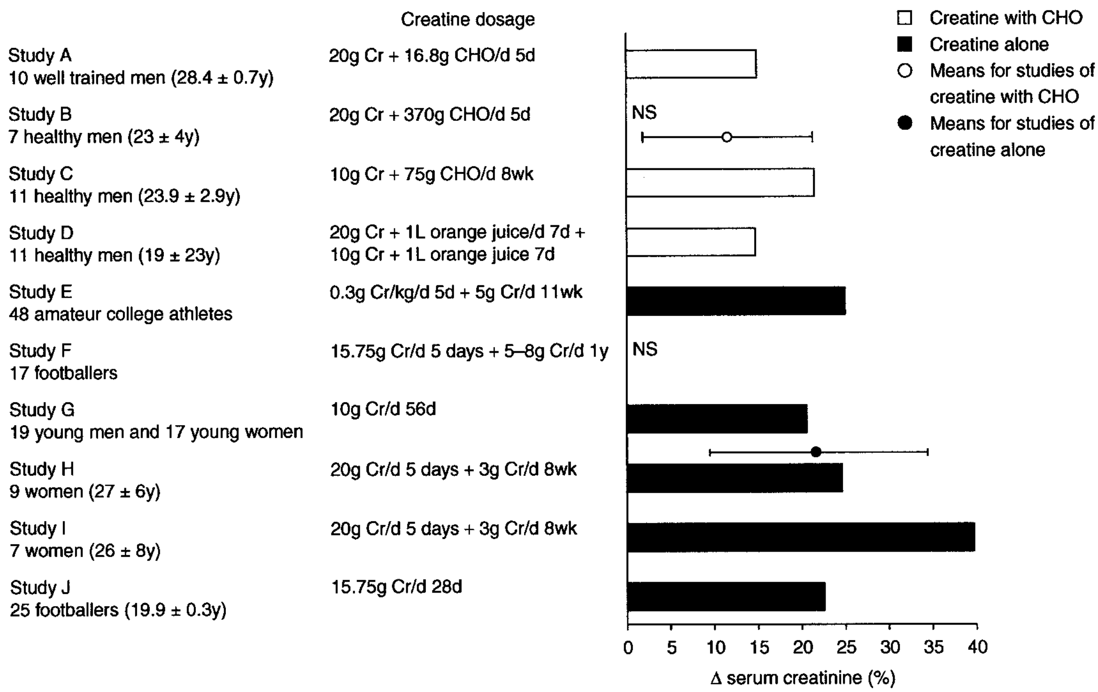

Fig. 13. Reported studies showing effects of creatine (Cr) supplementation with carbohydrates (CHO) and without CHO on serum creatinine values. Total studies of creatine supplementation are expressed as means and 95% CIs (error bars). Serum creatinine after creatine supplementation is higher in studies without CHO administration in conjunction with creatine.[123-131] **Study A** = Kamber et al.;[123] **Study B** = Robinson et al.;[124] **Study C** = Tarnopolsky et al.;[125] **Study D** = Jowko et al.;[126] **Study E** = Millard-Stafford et al.;[127] **Study F** = Kreider et al.;[128] **Study G** = Kuehl et al.;[129] **Study H** = Robinson et al.;[124] **Study I** = Robinson et al.;[124] **Study J** = Kreider et al.[130] **NS** = not significant; Δ indicates change in.

fore, less creatine remains in the circulation and less is non-enzymatically converted to Crn. This hypothesis is confirmed by the smaller increases in urinary Crn when creatine (20g creatine per day for 5 days[123] or 10g creatine per day for 8 weeks[125]) is administered with carbohydrates.

Similarly, Hultman et al.[122] reported that Crn excretion increased from ~10 to ~15 mmol/d after creatine administration alone (20g creatine/d, for 6 days). Indeed, after the first day of creatine supplementation, Crn excretion remained at baseline values (~10 mmol/d). On day 3 Crn urinary excretion was ~12.5 mmol, and on day 5 it was ~15 mmol. This is in accord with another study.[131] By contrast, Burke et al.[132] reported that urinary Crn excretion was insignificant at the second day of 7 days of creatine administration at a rate of 0.1 g/kg lean body mass (6–8g creatine/d) in 20 well trained sportsmen. These data are in agreement with earlier studies, which demonstrated that there is no increase in Crn excretion until a significant amount of the administered creatine has been retained.[2,3] In other words, Crn excretion increases only when TCr uptake is saturated. Taken together, these findings suggest that creatine administrated with carbohydrates favours its cell uptake, and therefore, less creatine is kept in the blood and excreted in urine. The best scientific evidence suggesting the drop in urinary creatine and Crn by creatine administration in conjunction with carbohydrates compared with creatine administration alone is the study of Steenge and co-workers.[133] In this study, the total urinary creatine and Crn excreted over a 40-hour period was less during and after a 24-hour creatine supplementation period (total of 20g, four times 5g creatine) in conjunction with carbohy-

drates. Moreover, creatine retention in muscle was higher after this kind of supplementation compared with placebo.

2.2.3 Return to Baseline Values

After cessation of creatine administration (20 g/d, for 6 days) TCr concentration in muscle gradually declines. Thirty days after cessation, the concentration is not different from the presupplementation values, and creatine loss from muscle during this period closely matches the increase in Crn excretion.[122]

2.3 Factors Affecting Creatine Uptake by Muscle Cells

Since 1920, creatine ingestion was recognised as an effective method to increase muscle TCr content.[4-7] Short-term creatine ingestion (20–30g creatine per day, for 5–10 days) has been shown to increase muscle TCr content by 15–20% in humans (~20 mmol/kg dm), with an upper limit of intramuscular TCr content at ~165 mmol/kg dm.[30,78,84,122,134-137] However, it is important to note that there is wide variation among individuals (0–40 mmol/kg dm).[30,134,138] This variation in creatine accumulation during supplementation can be accounted for by several factors, as discussed below.

2.3.1 Insulin Action

It has been reported in animal[32,139-141] and human[119,134] studies that an increase in muscle creatine accumulation originates from carbohydrate-mediated insulin release. In this respect, Green et al.[134] reported that creatine ingestion in combination with a carbohydrate-containing solution (93g of simple carbohydrates) increased muscle TCr by >25% in humans. This was 60% greater than the increase observed when creatine was ingested alone. In addition, creatine and carbohydrate ingestion dramatically elevated insulin concentration. Furthermore, when creatine was administered with simple carbohydrates the interindividual variability in muscle creatine accumulation was reduced: all participants showed an increase in muscle TCr content >20 mmol/kg dm; by contrast, only half of the individuals who ingested creatine alone had an increase of this magnitude (figure 14). This

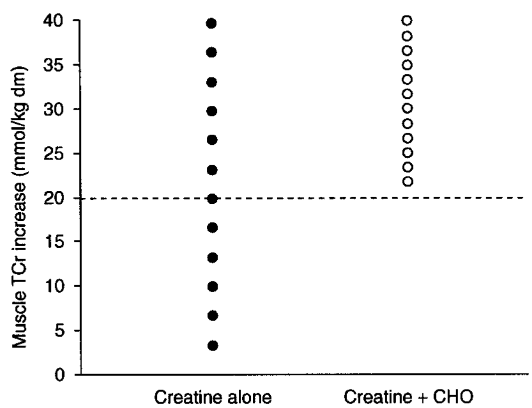

Fig. 14. Individual muscle total creatine (TCr) increase after ingesting 5g creatine alone or 5g creatine followed 30 minutes later by 93g simple carbohydrate (CHO) solution, four times each day for 5 days. Ingestion of creatine in conjunction with CHO reduced the interindividual variability in the magnitude of muscle TCr accumulation, such that all individuals demonstrated an increase in muscle TCr content >20 mmol/kg dm.[134] **dm** = dry mass.

study clearly suggests that an increase in muscle creatine accumulation originates from carbohydrate-mediated insulin release in humans. This is supported by a report showing that the AUC of plasma creatine and peak creatine concentration are lower after ingesting 5g creatine per day with 500ml of Lucozade (Smithkline Beecham, Coleford, UK) containing 94g of simple carbohydrates.[133] In this study, creatine retention was also higher compared with creatine administration without carbohydrates. The best scientific evidence suggesting the insulin-induced increase in creatine uptake by tissues is the study of Steenge et al.[142] These investigators found a strong correlation between the dose of infused insulin and the observed change in muscle TCr concentration ($r = 0.977$, $p < 0.001$) after a single creatine administration (12.4g). Two mechanisms have been proposed to explain this insulin-induced improvement in creatine uptake by tissues:

Insulin-Stimulated Muscle Blood Flow

Insulin stimulates muscle blood flow.[143] Consequently, the increase in muscle creatine accumulation after creatine and carbohydrate consumption in humans could be, at least in part, the result

of an insulin-mediated increase in muscle blood flow and thereby muscle creatine availability. This hypothesis was tested by Steenge et al.[142] in seven healthy men after ingestion of 12.4g creatine coupled with intravenous insulin infusion at four different rates. Insulin augmented muscle TCr content only when insulin was clamped at 100 and 200 mU/L (55 and 105 mU/m²/min), but no relation was observed between the insulin-induced increase in muscle blood flow and the increase in muscle TCr concentration at each insulin infusion rate (figure 15). Therefore, the contribution of insulin-mediated increases in muscle blood flow to stimulate muscle creatine uptake may be considered of little significance.

Insulin-Stimulated Na^+-K^+ ATPase

It has been reported that insulin translocates the Na^+-K^+ ATPase subunits, α2 and β1, from intracellular compartments to the plasma membrane.[144] This stimulates Na^+-K^+ ATPase function,[32] and allows creatine to enter the muscle against a concentration gradient.[22,32,35] Therefore, the insulin-induced enhanced TCr accumulation after creatine administration in conjunction with carbohydrates may be explained, at least in part, by the insulin-mediated translocation of Na^+-K^+ ATPase. Nevertheless, in the study of Steenge et al.[142] the insulin-mediated improvement in muscle creatine accumulation in humans was only present at high physiological (~100 mUI/L) or supraphysiological (~200 mUI/L) concentrations (55 and 105 mU/m²/min) [figure 15].

In summary, creatine accumulation can be substantially augmented in human skeletal muscle when creatine is ingested in conjunction with large quantities of simple carbohydrates able to stimulate endogenous insulin secretion. The contribution of insulin-mediated increase in muscle blood flow to stimulate muscle creatine uptake may be considered to be of little significance. Further research is therefore needed to clarify the amount of insulin and carbohydrates needed to maximise creatine uptake by muscle tissue.

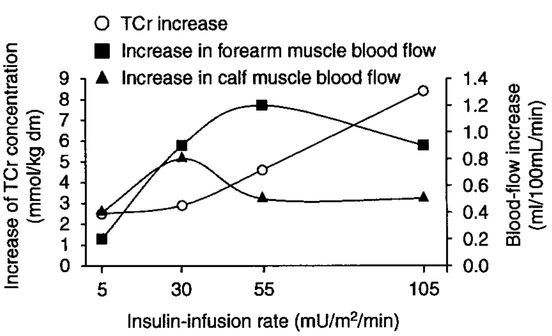

Fig. 15. Increase in regional blood flow and total creatine (TCr = creatine + phosphocreatine) content in vastus lateralis muscle, measured in seven healthy men (age 25.9 ± 3.0y, weight 72.6 ± 3.4kg) after ingestion of 12.4g creatine (oral and nasogastric administration) coupled with intravenous insulin infusion (at four different rates). Insulin augmented muscle TCr content only when insulin was clamped at 100 and 200 mU/L (55 and 105 mU/m²/min). No relationship was observed between the increase in muscle blood flow and the increase in muscle TCr concentration at each insulin infusion rate. Therefore, the contribution of insulin-mediated increase in muscle blood flow to stimulate muscle creatine uptake was insignificant.[142] **dm** = dry mass.

2.3.2 Intracellular Creatine Content

A recent study[145] analysed the changes in tissue creatine concentrations accompanying creatine administration (1–2g creatine/kg bodyweight/day for 8 weeks) in various animal species (rat, mouse and guinea pig). During creatine supplementation, the relative increase of TCr was low (15–55% of presupplementation values) in organs with high presupplementation concentrations (brain, skeletal and heart muscle, 10–22 mmol/kg wet weight), and high (260–500% of presupplementation values) in organs with low presupplementation concentrations (liver, kidney and lung, 5–8 mmol/kg wet weight). This suggests that the intra-extracellular creatine concentration gradient is another important determinant of creatine uptake. This is in agreement with data reported in humans, where the highest increases in muscle TCr after creatine supplementation are seen in individuals with low or depleted muscle TCr content.[30,84,146,147] This effect can be probably achieved by downregulation of creatine transporters expression.[35] These findings suggest that in order to maximise creatine uptake, a low presupplementation concentration of creatine in tissues is necessary. In fact, the increase in muscle TCr after creatine supplementation is higher in middle-aged

(increase by 30%) than in young people (increase by 15%), probably because the former have lower initial muscle TCr concentrations than younger individuals.[74] In agreement with this, Greenhaff et al.[84] reported that all of those who responded to creatine ingestion with increased muscle creatine content presented initial TCr concentrations lower than 120 mmol/kg dm, while those who did not respond had initial concentrations higher than 130 mmol/kg dm. These results may help to explain the large variability in muscle TCr reported in the literature after creatine administration.

2.3.3 Extracellular Creatine Concentration

It has been demonstrated that a high extracellular creatine concentration downregulates creatine transport into isolated skeletal muscle[148] by downregulated expression of gene CRT1 in muscle fibres.[149] By contrast, *in vitro* studies[150,151] have reported increased insulin secretion mediated by extracellular creatine content. Since insulin stimulates the Na^+-K^+ ATPase, this could offset the downregulation expression of gene CRT1, but *in vivo* studies[133,134,142] have failed to find any creatine-mediated increase in serum insulin. This topic therefore needs to be clarified.

2.3.4 Electrolyte Concentrations

In vitro studies have reported that extracellular electrolyte concentrations influence creatine uptake by muscle cells. In fact, this uptake augments as a hyperbolic function of extracellular [Cl^-] and as a sigmoid function of extracellular [Na^+].[34] In accordance with this, it has been reported that a decrease of extracellular [Na^+] from 145 to 25 mmol/L reduces the creatine uptake of type I muscle fibres by 77%.[152] Nevertheless, to our knowledge, there is not any evidence of *in vivo* studies suggesting such dependence. Since the variability on extracellular [Cl^-] and [Na^+] *in vivo* is minimal (much less than in experimental *in vitro* studies) and bearing in mind the equation relating extracellular [Cl^-] and [Na^+] changes with creatine uptake by muscle cells in *in vitro* studies, it is unlikely that changes *in vivo* on extracellular [Cl^-] and [Na^+] are able to affect creatine uptake by muscle cells.

2.3.5 Type of Fibre

After creatine administration in humans, creatine and PCr contents augment in both type I and II muscle fibres. However, a trend towards a larger increase in type II muscle fibres has been reported.[135,153] Since TCr content is higher in type II than in type I muscle fibres,[63-67,70] this is apparently in disagreement with the intracellular creatine content theory (see section 2.4.2), which suggests a lower increase in cells with a higher creatine content. These contradictory findings may be explained by a higher CRT1 expression in type II muscle fibres, offsetting in these cells the higher creatine content-mediated decrease in creatine uptake. A recent study[154] confirmed the higher uptake of creatine by type II muscle fibres. The authors found that old individuals (70 ± 2.9 years) had a relatively small increase (young 35% *vs* old 7%) in muscle PCr after creatine supplementation (20 g/d, for 5 days), probably because old individuals have fewer type II muscle fibres.

2.3.6 Physical Exercise Before Creatine Ingestion

Because physical exercise enhances muscle blood flow, creatine uptake by muscle cells could be augmented by the increase in muscle blood flow. In fact, it has been reported that creatine ingestion after 1 hour of submaximal physical exercise augments muscle creatine accumulation by 10%, but marked variations among individuals were observed.[30] Another study[155] showed that the enhanced creatine accumulation after physical exercise is specific for the exercised muscle, while the non-exercised contralateral muscle of individuals who supplemented with both creatine plus carbohydrates did not increase to the same extent as the active muscle. Therefore, a submaximal physical exercise-induced increase on muscle blood flow stimulates creatine uptake by muscle cells, although this is outweighed by creatine in combination with large quantities of simple carbohydrates.[119,134] The magnitude of muscle creatine accumulation is reduced when creatine and carbohydrates are ingested after strenuous exercise, likely due to an exercise-induced blunting of insulin release.[156] Therefore, when creatine is ingested in combination with carbohydrates, it is not

recommended to do strenuous physical exertion before the ingestion.

2.3.7 Other Factors

Other factors may enhance or decrease creatine uptake by muscle cells, as outlined below:

Enhancement of Creatine Uptake

There are several substances that appear to enhance muscle creatine uptake. Odoom et al.[32] used a G8 mouse skeletal muscle cell line to demonstrate that most of these substances also stimulate the Na^+-K^+ ATPase like insulin. Some of these substances are: insulin-like growth factor I, thyroid hormone (T_3) [~70 μmol/L] and amylin (~60 nmol/L). The nonspecific β-agonist isoprenaline increased TCr content 40–60%, and the β_2-agonist clenbuterol increased TCr content by 30%.

Decrease in Creatine Uptake

The study of Odoom et al.[32] reported that the α_1-agonist methoxamine decreased TCr content by 30%, whereas the β-antagonist (i.e. atenolol, butaxamine and propranolol) caused a slight reduction (<10%) in TCr content. Ouabain also inhibits creatine uptake,[157] like cyclosporin, by altering surface expression of the creatine transporter.[158] Creatine analogues are synthetic substances experimentally used to induce muscle creatine depletion. The structural analogues 3-guanidinopropionate and DL-3-guanidinobutyrate inhibit creatine uptake by muscle cells by 80 and 84% relative to creatine, respectively.[8,32,152,159] Finally, it has been reported that creatine uptake decreases in cases of tocopherol deficiency,[159] and an impairment on performance when creatine is ingested with caffeine has been reported.[160] In this study, both creatine administration alone (0.5 g/kg/d) or with caffeine (0.5g creatine/kg bodyweight/day + 5mg caffeine/kg bodyweight/day) for 6 days increased muscle PCr concentration by 4-6%, but while creatine administration improved performance during intense intermittent exercise, creatine plus caffeine ingestion eliminated such an ergogenic effect. In agreement with this, Hespel et al.[161] have recently reported a shortening (~5%) in muscle relaxation time after creatine administration (20 g/d, for 4 days), but not after the same ingestion in conjunction with caffeine. These authors suggested that creatine administration in conjunction with caffeine overrides the shortening of muscle relaxation time caused by creatine supplementation. Since muscle relaxation time is an important factor in the performance of nonsustained muscle contractions (see section 3.5.4), caffeine ingestion in conjunction with creatine is not recommended to augment performance in this kind of exercise.

2.4 What is the Appropriate Creatine Administration Scheme for Optimal Muscle Uptake?

Creatine is usually administered as a dosage regimen consisting of a loading phase of 20 g/d (four times 5g) for 5–7 days and a maintenance dosage of 3–5 g/d thereafter. Based on creatine pharmacokinetics and the previous discussion, we propose the following scheme of creatine administration.

2.4.1 Optimal Single Dose

Creatine uptake by muscle cells presents Michaelis-Menten kinetics. The Michaelis constant (K_m) for this transport ranges from 15–30 μmol/L[35] (figure 16). Therefore, the maximal creatine uptake by muscle cells is probably produced at concentrations above 300–400 μmol/L. In fact, following 5g of oral creatine ingestion the peak value of serum creatine reaches plasma values of 600–800 μmol/L within 1 hour.[30,119] Considering K_m, this concentration is optimal to maximise creatine uptake by muscle cells. Moreover, this enhanced creatine uptake may even be improved by the simultaneous ingestion of simple carbohydrates. Nevertheless, in order to produce peak creatine and insulin concentrations at similar time-points, a 30-minute delay between creatine and a subsequent carbohydrate beverage (500ml with 90–100g of simple carbohydrates) should be allowed.[133,134]

2.4.2 Optimal Multiple Dosage During the First Day (Loading Phase)

A single creatine dose is not sufficient to guarantee adequate muscle stores, and subsequent administrations are therefore required. Because the return to fasting values after the previous creatine

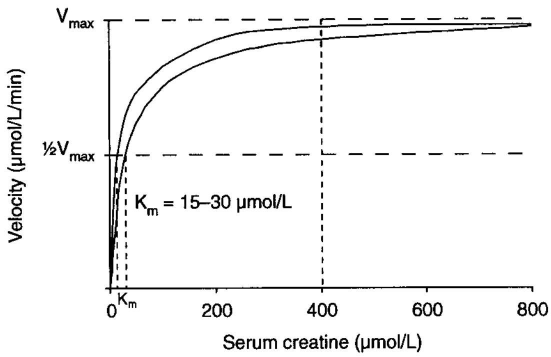

Fig. 16. Michaelis-Menten kinetics for creatine uptake by muscle cells. Creatine uptake by muscle cells shows an apparent Michaelis-Menten constant (K_m) for creatine in the micromolar range (15–30 μmol/L). Maximal rate of creatine uptake by muscle cells is therefore obtained at serum concentrations higher than 300–400 μmol/L.[35] V_{max} = maximal velocity.

dose occurs after 5–7 hours,[30,119] it seems necessary to administer four repeated doses of 5g separated by 5–7-hour intervals during the first day in order to maintain an increased plasma creatine concentration and improve creatine transport into muscle cells. Steenge et al.[133] studied the effects of multiple creatine administration (four times 5 g/d, for 1 day) alone and in conjunction with carbohydrates. Excreted creatine and Crn were less and creatine retention was higher after carbohydrate treatment. Therefore, the optimal creatine ingestion during the first day seems to be four repeated doses (separated by 5–7-hour intervals), of 5g creatine in conjunction with 500ml containing 90–100g of simple carbohydrates.

2.4.3 Optimal Multiple Dosage During the Second Day (Loading Phase)

With the use of urinary creatine excretion measurements and the assumption of a muscle mass equivalent to 40% of body mass, Steenge and coworkers[133] calculated that muscle TCr content increased by ~7 mmol/kg dm during the 24 hours of creatine administration combined with placebo solution. However, after carbohydrate treatment, the estimated muscle TCr content increased by ~9 mmol/kg dm. Nevertheless, these benefits are not sufficient to improve physical performance since it has been suggested that an increase in muscle TCr content in excess of 20 mmol/kg dm is required to exert an ergogenic effect on muscle power output[135] and postexercise PCr resynthesis.[84] Therefore, creatine administration must continue. The ingestion of ~100g of simple carbohydrates with each 5g creatine dose is close to the limit of palatability over several days of supplementation.[134] In this respect, it has been reported that the ingestion of creatine (four times 5g) in conjunction with 47g of simple carbohydrates and 50g of protein resulted in a similar increase in muscle TCr content than after the same creatine administration in conjunction with 94g of simple carbohydrates. Therefore, for the second day, the ingestion of four times 5g creatine in conjunction with 47g of simple carbohydrates and 50g of protein with each creatine dose can be recommended.

2.4.4 Optimal Dosage Thereafter (Maintenance Phase)

The majority of muscle creatine accumulation occurs in the initial 2 days and, as total creatine accumulation is maximised, in the subsequent days there is an increase in serum creatine and Crn, with spillover of both molecules in the urine (see section 2.2.1 and section 2.2.2). In fact, it has been reported that creatine in the urine increases from 214 ± 44 to 1580 ± 159 arbitrary units after 20g creatine per day for 5 days even when administered in conjunction with carbohydrates.[123] In addition, Steenge et al.[133] reported an increase in urinary creatine after four times 5g creatine during 1 day in conjunction with carbohydrates, this increase being lower than when creatine administration was performed alone. This creatine spillover in the urine suggests that transport and accumulation of creatine into muscle cells is almost saturated even during the first day of creatine administration in conjunction with carbohydrates. These data suggest that creatine accumulation is maximised in the initial 2 days of creatine administration even when administered in conjunction with simple carbohydrates. Therefore, the maintenance phase would start on day 3 of creatine administration. For this phase, 3–5 g/d in conjunction with carbohydrates seems reasonable. Since the bulk of data suggest that creatine has a saturating effect on the muscle, we could hypothesise that once loading is accom-

plished, the ingestion of creatine three to four times per week, instead of daily, could be sufficient to maintain total muscle creatine, given the high degree of muscle saturation and the prolonged washout effects. Though this has not been proven – or even examined – we believe that it is something that is both reasonable and a potential topic for future research.

In summary, based on creatine pharmacokinetics, a supplementation regimen integrated by a loading phase of 20 g/d (four times 5g) for 2 days and a maintenance dose of 3–5 g/d thereafter may be proposed. During the first day of the loading phase, creatine must be ingested in conjunction with simple carbohydrates (500ml containing 90–100g of simple carbohydrates), whereas on the second day, creatine could be ingested in conjunction with 40–50g of simple carbohydrates and 50g protein. The maintenance dose can be of 3–5g creatine in conjunction with simple carbohydrates. Carbohydrate beverage administration should be ideally ingested after 30 minutes of creatine ingestion to produce peak creatine and insulin concentrations at similar time points in order to stimulate muscle creatine accumulation. We hypothesise that this kind of creatine administration increases muscle TCr content at least as rapidly as with classical creatine administration but without creatine and Crn spillover in the serum and urine. The ergogenic effect of this creatine supplementation is the object of further discussion.

3. Creatine Administration as an Ergogenic Aid

Reports of improved performance and weight gain in animals and humans after creatine ingestion date back to studies conducted in the early 1900s.[1-7] After one century of these findings creatine is probably the most popular and effective ergogenic aid presently available. In the US, creatine consumption over the last 5 years has mounted to over 2 million kilograms per year.

3.1 Usual Dose of Creatine Administration

To evaluate the effects of creatine supplementation, a commonly used dosage of 20 g/d of creatine ingested for 4–6 days is employed; this represents a dose of ~0.3g creatine/kg bodyweight/day for an average adult. Generally, this amount is divided in 5g doses, four to five times per day. With this supplementation, most studies report an average response of a 15–20% increase in muscle TCr content.[30,122,135,155] Such an increase is sufficient to exert an ergogenic effect on muscle power output[61] and postexercise PCr resynthesis.[84]

Indeed, muscle creatine increases by ~50% and muscle PCr by 12.5%,[162] so the PCr/creatine ratio is lower[30,122,136] and the PCr/ATP ratio at rest and muscle TCr are higher than before supplementation.[163]

3.2 Creatine Supplementation and Short-Term High-Intensity Exercise

A classical study,[164] published in 1981, suggested that creatine supplementation is an efficient method for increasing strength and athletic performance during short-term high-intensity exercise. In this study, seven patients ingested daily 1.5g of creatine for 1 year. The patients increased body mass in about 10% and several individuals improved their strength. One patient, who was an active runner, improved his 100m mark by above 10%, reducing it from 17 to 15 seconds. More recently, numerous studies[126,130,131,137,165-183] have confirmed the ergogenic effects of creatine administration on short-term high-intensity exercise, mainly at dosages of 15–25 g/d for 5–10 days followed by 3–5 g/d thereafter. Even with lower dosages (7.7g creatine per day, for 21 days), it has been reported to have an ergogenic effect on muscle output.[184] Performance in isometric exercise is augmented by about 15% in isotonic, and by about 10% in isokinetic work. In these studies, differences were reported in age but not in gender. Consequently, elderly people (>60 years) may improve slightly in isokinetic strength after creatine supplementation.[185,186]

By contrast, there are several studies that do not show any ergogenic effect on high-intensity exercise after creatine supplementation.[156,187-196] In these studies, probably, muscle TCr content did not increase in excess of 20 mmol/kg dm. As suggested, this increment is required to exert an

ergogenic effect on muscle power output.[135] In addition, several of these studies were performed with elite athletes (in whom it is more difficult to enhance muscle output),[187,188,191] in a small number of individuals,[189,190,194] over short periods of creatine administration,[192] with no habitual conditions for the sport,[193] and used exercises that measured coordination but not specifically strength.[195] Therefore, most of the double-blind cross-over designs and double-blind studies suggested an ergogenic effect of creatine administration on muscle output for short-term high-intensity exercise. Nevertheless, more research is needed to clarify the ergogenic effects of lower dosages of creatine administration. Several physiological mechanisms may underlie the ergogenic action of creatine supplementation in short-term high-intensity exercise.

3.2.1 Increase of ATP Availability

As previously mentioned (see section 1.5.1), during a 6-second sprint at a power output representing 250% $\dot{V}O_{2max}$, PCr hydrolysis contributes 50% of the total ATP, whereas during a 30-second sprint at 200% $\dot{V}O_{2max}$, PCr hydrolysis contributes 25% of the total ATP requirement. It is well known that muscle PCr content is increased after creatine supplementation. If PCr is increased 10–20% after creatine administration, the energy supply will be increased by 5–10% and 2.5–5%, for the 30- and 6-second sprints, respectively.[86] Thus, creatine supplementation may be of potential benefit in energy provision during short-term high-intensity exercise, because of its buffering cytosolic phosphorylation potential.[197] Since the energy cost of muscle contraction does not vary after creatine supplementation,[198] the muscle power output should be higher, and this is mainly seen in type II muscle fibres.[102] This theory is currently accepted and it has been advocated as an explanation for the success of some sprinters.[199]

3.2.2 Muscle Fibre Hypertrophy

Volek et al.[137] reported that creatine supplementation for 12 weeks (25 g/d for the first week followed 5 g/d thereafter) in conjunction with heavy resistance training increased type I, IIA and IIAB muscle fibre cross-sectional area by 11, 35 and 36%, respectively. By contrast, in the placebo group (only heavy resistance training), these cross-sectional areas increased by 11, 15 and 6%, respectively. Other data[200] suggest that oral creatine supplementation may augment fibre hypertrophy during a resistance-training programme following muscle disuse atrophy. Accordingly, it has been reported that ingestion of 20g creatine per day for 5 days, in conjunction with resistance training, increased arm cross-sectional area by 7.9cm^2 versus placebo.[171] Taken together, these findings suggest that creatine administration in conjunction with resistance training results in greater muscle fibre hypertrophy than training alone. There are three possible physiological mechanisms underlying this muscle fibre hypertrophy, as outlined below:

Growth Hormone Delivery

It has been suggested that creatine has an indirect anabolic effect.[201] In this comparative cross-sectional study,[201] a significantly higher growth hormone level was observed after acute creatine loading (20g). Nevertheless, a large interindividual variability was observed. The peak plasma values of growth hormone were generally obtained 2–6 hours after creatine administration. In another report,[202] creatine ingestion did not affect either growth hormone nor testosterone secretion. In this study, venous blood was sampled before, immediately after, and 30 and 60 minutes after the training session, but no measures were done 2–6 hours after creatine administration, when the potential indirect anabolic effect of creatine is higher. Therefore, this mechanism is not clear at present and further research is warranted.

Contractile Protein Synthesis

Ingwall et al.[203] showed that creatine, in a concentration range between 10 μmol/L and 5 mmol/L, significantly increased the synthesis of myosin heavy chain protein *in vitro*. Accordingly, Silber et al.[204] reported that 5 mmol/L of exogenous creatine added to the culture of growing myoblasts stimulated selectively the biosynthesis of the specific contractile protein myosin heavy chain by 30%. Subsequent studies have confirmed these results,[205-209] which have been supported by the fact that animals fed with the creatine analogue β-guanidinopropionic acid, which induces a deple-

tion in intramuscular creatine, experience a loss of myofilaments and hypotrophy of type II muscle fibres.[210-212] Nevertheless, these studies did not attempt to determine an underlying mechanism but speculated that creatine may actually increase myosin synthesis. In this regard, Willoughby and Rosene[213] showed a 57.92% increase in myofibrillar protein after 12 weeks of creatine administration at a rate of 6g creatine per day in conjunction with resistance training. This increase was significantly higher than that observed in the control (2.75%) and placebo (11.62%) groups. The authors suggested that the increased expression of myosin heavy chain mRNA and protein as a result of 12 weeks of creatine supplementation were reflected in the observed increase in myofibrillar protein content. Since this study incorporated participants who were not resistance trained, further research with resistance-trained individuals is necessary.

In addition, it has been reported that combination of creatine and increased functional loading augmented satellite cell mitotic activity.[214] When creatine accumulates in cells, water drag occurs and results in increased cell hydration. In fact, an increase of 2–3% in intracellular fluid volume has been reported after creatine ingestion (0.35g creatine/kg fat free mass/d).[215] Hyperhydration may act as an anabolic signal stimulating protein synthesis,[28] but this theory has not been directly investigated. It has to be stated that other investigators failed to show any stimulatory effect of creatine on protein synthesis.[216-219] However, most of these studies were *in vitro* or animal models, not representative of an *in vivo* human model like the study of Willoughby and Rosene.[213] Therefore, taken together, these data suggest an anabolic property of creatine, by an increase in myosin heavy chain isoform mRNA expression, mainly when creatine is administered in conjunction with resistance training. However, this property remains to be demonstrated in resistance-trained individuals.

Reduced Protein Catabolism

An anticatabolic effect of creatine administration could also explain, at least in part, the potential hypertrophic effect of creatine. In the series of experiments by Ziegenfuss et al.,[215] creatine was able to improve net nitrogen status by increasing protein synthesis and/or decreasing protein breakdown. Moreover, using whole-body leucine kinetics and mixed muscle fractional protein synthetic rates, Parise et al.[219,220] measured protein synthesis during creatine supplementation in humans. They did not find any increase in protein synthesis, but suggested a possible decrease in protein catabolism in men, but not in women. Whether creatine may decrease protein breakdown remains to be elucidated.

In summary, the ergogenic effects of creatine supplementation on muscle power output are mainly caused by both increased ATP availability and muscle hypertrophy. This last effect is probably mediated by a creatine-mediated increase in myosin heavy chain isoform mRNA expression.

3.3 Creatine Supplementation and Mid-Term High-Intensity Exercise

There are few studies evaluating the effects of creatine supplementation on maximal single exercises lasting 30–140 seconds. One study has reported that creatine supplementation increased anaerobic capacity and maximum accumulated oxygen deficit (MAOD).[221] In this double-blind study, 14 individuals exercised to exhaustion at 125% $\dot{V}O_{2max}$ after creatine supplementation (20 g/d, 5 days). MAOD increased after creatine administration from 4.04 ± 0.31 to 4.41 ± 0.34L and remained elevated for another 7 days. Time to exhaustion also increased after creatine administration from 130 ± 7 to 141 ± 7 seconds and remained increased for another 7 days. Accordingly, it has been reported that creatine supplementation (20 g/d, for 5 days) increased, by some 15%, muscle work output in three maximal tests of 90, 150, and 300 seconds, performed by elite kayakists.[222] Viru et al.[223] obtained similar results in middle-distance runners. Similarly, creatine supplementation (20 g/d, for 5 days) increased the performance in jumping test (30 seconds) by 7% for the first 15 seconds, and by 12% for the last 15 seconds.[168] The leg time limit to exhaustion at 80, 60, 40 and 20% of maximal voluntary isometric contraction was increased in resistance-trained men after cre-

atine supplementation (10 g/d, for 5 days).[177] Thus, the ergogenic effect of creatine supplementation on lactic metabolism seems evident. However, more research is needed on this topic to confirm this ergogenic effect of creatine on high-intensity exercise, since there are few studies regarding this topic. Possible mechanisms underlying this ergogenic effect may be the increase in ATP availability and enhanced muscle hypertrophy after creatine supplementation (see section 3.2.1 and section 3.2.2). In addition, the decrease of ammonia and hypoxanthine in muscle and plasma (see section 3.4.2) and pH buffering of creatine ingestion could play a significant role, since: (i) a higher muscle PCr content produces higher [ATP] buffering (and consequently activation of glycogenolysis is delayed); and (ii) PCr hydrolysis contributes to buffering pH (since the reaction uses H^+) [figure 3]. These assumptions have recently been confirmed by Roussel et al.,[224] who found a linear relationship ($r = 0.61$, $p = 0.0007$) between PCr resynthesis, PCr hydrolysis, and pH at the end of exercise.

In summary, although further research is needed (mainly concerning gender and age), the ergogenic effect of creatine supplementation on high-intensity exercise (from 30–140 seconds) appears evident, even in well trained individuals.

3.4 Creatine Supplementation and Intermittent High-Intensity Exercise

Intermittent high-intensity exercise is the usual form of exercise present in most sports. Numerous studies have reported that creatine supplementation at a rate of 15–25 g/d for 4–8 days improved performance in intermittent high-intensity exercise even in well-trained individuals.[123,168,170,182,225-235] More recent studies confirm this assumption.[131,162,165,169,236] Skare et al.[169] reported a slight improvement in the total time of six intermittent 60m sprints (45.63 ± 1.11 vs 45.12 ± 1.1 seconds) in well-trained men after classical creatine administration (20 g/d), whereas no changes were observed in the placebo group. The sprint velocity was significantly increased in five out of six intermittent 60m sprints. Preen et al.[162] reported that creatine supplementation improved performance during 80 minutes of repeated sprints, and the total work production increased by 6%.

In the study by Romer et al.,[165] nine competitive squash players performed an on-court 'ghosting' routine that involved 10 sets of 2 repetitions of simulated positional play, each set interspersed with 30 seconds of passive recovery. Creatine at a rate of 0.3 g/kg body mass/d for 5 days was administered in a double-blind and crossover fashion. Sets 2 to 10 were completed in a significantly shorter time following creatine supplementation compared with the placebo condition. In another recent study,[131] creatine supplementation (20 g/d, for 5 days) significantly increased the average running times during the first 5m of repeated 15m sprints (3%) in nine trained male handball players. Moreover, creatine ingestion improved the number of repetitions prior to fatigue, and the total average power output values during repetitive high-power output exercise bouts in bench press and half squat. Rossouw et al.[236] reported an improvement in performance of 3 sets of maximal unilateral knee extensions on an isokinetic dynamometer interspaced with 60-second rest periods in eight well-trained power-lifters. In addition, these authors also reported improvement in a maximal deadlift strength feat performed in a gymnasium. Cottrell et al.[237] also found an improvement in performance on intermittent high-intensity exercise (cycling) in adult men after creatine supplementation (0.3 g/kg body mass/d, for 7 days). One study[238] reported that acute loading (25 g/d, for 5 days) was more effective than chronic loading (5 g/d, for 60 days) in elite swimmers. These benefits are even maintained after exercise-induced previous exhaustion.[239,240]

Several studies have not reported any ergogenic effect of creatine supplementation in performance of intermittent high-intensity exercise.[241-244] Several shortcomings may offset the ergogenic effect of creatine supplementation in these studies. In the study of Barnett et al.,[241] muscle TCr content probably did not increase in excess of 20 mmol/kg dm, and this increment is probably required to exert an enhanced rate of PCr resynthesis during recovery.[84] In the study of McKenna et al.,[244] 30g

creatine per day for 5 days increased muscle TCr by 22.9 ± 4.2 mmol/kg dm (sufficient to improve performance in intermittent high-intensity exercise), but it did not improve performance in five 10-second maximal cycle ergometer sprints with rest intervals of 180, 50, 20 and 20 seconds. In this study, performance (both peak power output and cumulative work production during maximal intermittent exercise) improved, but not significantly. Inclusion of higher numbers of participants would help to clarify this issue. The study by Cooke and Barnes[243] also used a small sample (eight participants) in the creatine group. In the study of Gilliam et al.,[242] the investigators reported that creatine administration at a rate of 20 g/d for 5 days did not improve performance in 5 sets of 30 maximum volitional isokinetic contractions with a 1-minute rest period between sets in untrained men. This conclusion came after a three-way mixed ANOVA with one between factor (placebo vs creatine) and two within factors (pre/post supplementation and sets 1–5). However, the pre/post and set main effects were significant, increasing peak torque (approximately 3%) from pre- to post-testing. This may indicate a learning effect in both groups. However, the possibility that a learning effect occurred only for the placebo group cannot be excluded, whereas a creatine-induced ergogenic effect occurred only for the creatine group. Taken together, these findings suggest that creatine administration (15–25 g/d, for 4–8 days) improves performance in intermittent high-intensity exercise even in well-trained individuals. Several mechanisms related to higher ATP availability and muscle fibre hypertrophy (see section 3.2.1 and section 3.2.2) may explain the ergogenic effect of creatine supplementation on intermittent high-intensity exercise.

3.4.1 Enhanced Rephosphorylation Rate of ADP into ATP

During intermittent high-intensity exercise, each period of short-term high-intensity exercise is followed by a rest period. Because intramuscular ATP and PCr are partially depleted during periods of high-intensity exercise, rapid restoration of intramuscular stores of ATP and PCr is needed in order to increase performance. In this regard, it is well known that after creatine ingestion (20–30g creatine per day, for 5–7 days), an increase in muscle TCr content occurs. Indeed, this increase is mainly in the form of free creatine (50% of the increase) and secondarily in the form of PCr (12.5% of the increase).[162] Both free creatine and PCr increases may have beneficial effects on the rephosphorylation rate of ADP into ATP. The excess of muscle PCr content induced by creatine supplementation augments the rephosphorylation rate of ADP into ATP,[84,162] In addition, part of the excess of free creatine stored in muscle after creatine supplementation may diffuse to the intermembrane space of mitochondria, where it is phosphorylated to PCr. This PCr may leave the mitochondria and diffuse through the cytosol to the sites of ATP consumption. Indeed, a significant relationship between the percentage increase in TCr content after supplementation versus the percentage change in PCr after 2 minutes of recovery has been reported.[196] The increase in PCr resynthesis occurs because of an increase in mitochondrial ATP production, which is regulated by the availability of mitochondrial ADP (figure 9). It has been hypothesised that both mitochondrial ADP formation and ATP resynthesis are linked to the phosphorylation of free creatine at the intermembrane space of mitochondria.[8]

Since it is likely that the free creatine concentration is higher in type II muscle fibres after creatine supplementation (see section 2.3.5), creatine supplementation may improve intermittent high-intensity exercise by increasing mitochondrial ATP and PCr production in these fibres. Consequently, PCr resynthesis is augmented after creatine supplementation, but an enhanced rate of PCr resynthesis during recovery may only occur in individuals who display a marked increase in TCr (in excess of 20 mmol/kg dm) after creatine supplementation.[84,196] In fact, it has been reported that creatine supplementation (21 g/d, for 14 days) increased muscle PCr reposition by 15 and 10% after 50 ankle flexo-extensions at 40 and 70% of maximal voluntary contractions, respectively.[245] Greenhaff et al.[84] reported that creatine supplementation (20 g/d, for 5 days) increased muscle PCr reposition by 20% after 2 minutes of recovery

from isometric high-intensity exercise. The optimal reposition time of muscle PCr is longer than 20 seconds.[246] Rest periods less than 20 seconds are insufficient to restore muscle PCr and ATP stores even after creatine supplementation. The higher PCr and ATP restoration in intermittent high-intensity exercise allows a higher muscle power output during short-term periods of high-intensity exercise and a higher muscle ATP and PCr restoration during rest intervals. Therefore, through enhanced rephosphorylation rate of ADP into ATP, creatine supplementation may contribute to improve performance in intermittent high-intensity exercise. Nevertheless, further research will clarify mechanisms underlying such an improvement.

3.4.2 Reduction in Ammonia and Hypoxanthines in Muscle

During short-term periods of high-intensity exercise, the first buffer of muscle ATP content is PCr hydrolysis. The subsequent decrease in ATP provision may lead to a transient increase in muscle ADP content, which stimulates the adenylate kinase reaction:

2 ADP ↔ATP + AMP

This reaction results in the formation of AMP, which is rapidly deaminated to inosine monophosphate (IMP) and ammonia via the activity of AMP deaminase,[247] and IMP is transformed to hypoxanthine (figure 17). Thus, it is well known that periods of high-intensity exercise result in a reduction in the muscle total adenine nucleotide pool ([ATP] + [ADP] + [AMP]) and an increase in IMP, ammonia, and hypoxanthine concentrations.[248-250] In fact, ammonia and hypoxanthine plasma levels are considered markers of adenine nucleotide loss in high-intensity exercise.[251] Such a reduction in the muscle total adenine nucleotide pool and the increase in IMP, ammonia, and hypoxanthine lead to an impairment of intermittent high-intensity exercise performance. Creatine supplementation may offset this impairment. Greenhaff et al.[138] reported that an increase in muscle PCr (after creatine supplementation) buffered muscle ATP levels, and consequently the accumulation of ammonia and hypoxanthines in plasma was reduced and the muscle power output increased. Several other studies confirm this hypothesis.[191,248,249] It has even been shown that creatine supplementation (20 g/d, for 5 days) significantly reduced blood ammonia concentration in six male football players during thirty 5-second maximal riding sessions (load: 7.5% bodyweight kiloponds) on a cycle ergometer interspersed with 90-second no-load exercise (80 rpm).[252] Additionally, mean power output significantly increased after creatine supplementation.

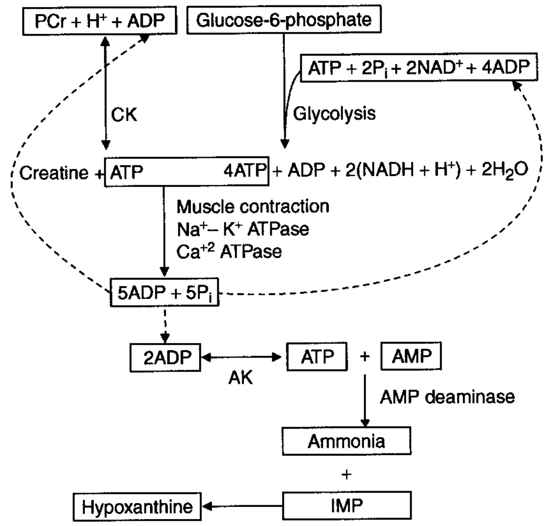

Fig. 17. Muscle total adenine nucleotide metabolism during short-term high-intensity exercise. During short-term high-intensity exercise as a 6-second sprint, phosphocreatine (PCr) hydrolysis and glycolysis each contribute ~50% of the total ATP requirement. This exercise results in a reduction in the muscle total adenine nucleotide pool ([ATP] + [ADP] + [AMP], where brackets denote concentration) and an increase in inosine monophosphate (IMP), ammonia and hypoxanthine concentrations. After creatine supplementation, muscle PCr is higher and ATP produced by PCr hydrolysis is available for a longer time. This higher ATP availability results in a smaller increase in IMP, hypoxanthine and ammonia concentrations. Nevertheless, the increase in ATP availability by PCr hydrolysis may be offset by creatine supplementation–induced inhibition of glycolysis. Further research is therefore needed to clarify the optimal creatine administration and muscle total creatine increase to obtain a smaller increase in IMP, hypoxanthine and ammonia. **AK** = adenylate kinase; **CK** = creatine kinase; **NAD+** = oxidised nicotinamide-adenine dinucleotide; **NADH** = reduced nicotinamide-adenine dinucleotide; **Pi** = inorganic phosphate.

However, Snow et al.[196] reported that creatine supplementation increased muscle total TCr con-

tent by ~9.5%, but did not affect ammonia or hypoxanthine levels in plasma after high-intensity exercise. In this study, performance was unaffected after creatine supplementation. Therefore, for reducing ammonia and hypoxanthines in plasma after high-intensity exercise, muscle TCr content should probably be augmented above ~10%. Taken together, these findings suggest that creatine supplementation may induce a drop in ammonia and hypoxanthine in muscle. This may enhance performance in intermittent high-intensity exercise, although further research is required to clarify this issue and the necessary increase in muscle TCr to obtain such an improvement.

3.4.3 Reduction in Lactate and H^+ Accumulation

During short periods of high-intensity exercise, lactate and H^+ cations may be accumulated in muscle, originating fatigue. After creatine supplementation, muscle TCr is augmented and this excess of TCr may offset ATP and PCr depletion during high-intensity exercise, and therefore, offset lactate and H^+ accumulation in muscle. This would contribute to a delay in fatigue. Indeed, several studies have reported that high-intensity exercise-mediated lactate accumulation was lower after creatine supplementation.[78,123,136,226,235] Similarly, in these conditions, lactate production is not increased in spite of a higher work production.[227] By contrast, several studies have failed to show a lactate decrease after creatine supplementation.[131,253,254] Therefore, additional studies are needed to clarify whether creatine supplementation may reduce lactate accumulation in muscle during high-intensity exercise. Because PCr resynthesis is dependent on adequate blood flow and oxygen delivery to remove metabolites such as lactate and H^+,[96] creatine supplementation would be expected to enhance PCr resynthesis during intermittent exercise above ~70% of maximal voluntary contraction, because of the appearance of local ischaemia. For adequate ATP and PCr restoration, recovery would have to be initiated by a sudden return of blood flow to muscle. Several studies have suggested an improvement in ATP and PCr recovery after occluded exercising muscle,[63,95] so it is possible that creatine supplementation is able to improve ATP and PCr recovery during intervals of isometric exercise much more than during dynamic exercise. This hypothesis remains to be elucidated.

In summary, it seems probable that creatine supplementation may improve performance in intermittent high-intensity exercise by both enhancement of rephosphorylation rate of ADP into ATP and a reduction in ammonia and hypoxanthine levels in muscle during physical exertion.

3.5 Creatine Administration and Aerobic Metabolism in Muscle

It has been reported that creatine supplementation did not improve aerobic performance in either trained or untrained individuals,[131,255-258] partly because of creatine-induced increases in body mass, which consequently cause higher absolute rates of $\dot{V}O_2$. In contrast, it has been previously discussed that the extent of the increment of TCr appears to influence PCr levels after 2 minutes of recovery. These results indicate an improvement of oxidative phosphorylation after creatine supplementation because the resynthesis of PCr in humans occurs solely in the presence of oxygen.[8] Accordingly, several recent studies have shown that creatine supplementation may improve aerobic muscle metabolism. Rico-Sanz and Marco[259] have reported that creatine supplementation (20 g/d, for 5 days) increased the time to exhaustion from 30 ± 4 to 36.5 ± 6 minutes after bicycle submaximal exercise. Nelson et al.[260] found that creatine supplementation (20 g/d, for 7 days) improved the time to exhaustion in a graded cycle exercise test from 1217 ± 240 to 1289 ± 215 seconds. There are previous studies that confirm this assumption. Harris et al.[59] reported a reduction of 2.1 ± 0.6 seconds on four times 1000m running after creatine loading (30 g/d, for 6 days). Rossiter et al.[261] showed a decrease of 2.3 seconds on 1000m rowing in elite rowers after creatine ingestion (0.25 g/kg body mass/d, for 5 days). Smith et al.[262] found that creatine supplementation (20 g/d, for 5 days) increased the time to exhaustion (from 236 to 253 seconds) at 3.7 W/kg body mass on a cycloergometer. Furthermore, creatine ingestion (20 g/d, for 7 days) reduced plasma ammonia and hypoxanthine levels after endurance exercise.[263]

For obtaining these benefits, creatine supplementation must be above 2 g/d.[264] Because creatine supplementation is accompanied by an increment in body mass, higher improvements in endurance exercise are obtained in nonweightbearing endurance events. Several mechanisms may explain the improvement of creatine supplementation on aerobic performance:

3.5.1 Creatine Supplementation Buffers pH Drop

It has been reported that creatine supplementation (5 g/d, for 11 days) produced less utilisation of PCr and higher pH after repeated plantar flexion isometric exercise at 32% of maximal voluntary contraction.[265] In this study, H^+ accumulation was lower, suggesting that the rate of net H^+ production was also lower. Therefore, creatine supplementation may increase the anaerobic threshold. In fact, creatine loading (20 g/d, for 7 days) increased the anaerobic threshold from 2.2 to 2.5 L/min.[266] In addition, another study[267] showed a reduction in lactate response after 6 minutes of a cycloergometer task at an intensity above ventilatory threshold after creatine supplementation (20 g/d for 5 days, and 5 g/d thereafter).

3.5.2 Optimisation of Oxidative Phosphorylation

The enhancement of oxidative phosphorylation by creatine occurs via Mi-CK in the mitochondrial intermembrane space and via the creatine/PCr shuttle, which carries creatine to mitochondria and provides PCr to the myofibrils, sarcolemma and sarcoplasmic reticulum (figure 9). The Mi-CK system seems to be an essential constituent of energy metabolism,[268] since the ADP generated by Mi-CK is an important regulator of oxidative phosphorylation in skeletal muscle.[269] The Mi-CK proportion may be augmented by endurance training[270] or by chronic low-frequency electric stimulation.[271] In cardiomyocytes, the higher respiration rate and higher rate of PCr production observed after creatine addition were suggested to be caused by an amplification of the sensitivity of respiration to ADP,[272] because of the coupling of Mi-CK, adenine nucleotide translocase, and oxidative phosphorylation.[112]

3.5.3 Creatine-Induced Glycogen Supercompensation

High concentrations of muscle glycogen are necessary for performing in long-distance running.[273] It has been shown that glycogen supercompensation tends to be greater if creatine and glycogen are loaded simultaneously because creatine loading increases cell volumes and, therefore, enhances glycogen supercompensation.[155] Accordingly, it has been suggested that the muscle glycogen loading capacity is influenced by the initial creatine levels and the accompanying alterations in cell volume.[274] In this study, total glycogen content after creatine load (20 g/d, for 5 days) was significantly greater than before (694 ± 156 vs 597 ± 142 mmol/kg dm). This higher than average glycogen level after creatine supplementation is established above 650 mmol/kg dm.[275] The greater glucose availability after creatine supplementation may be explained by the increase in GLUT-4.[276,277] It has been suggested that this increase in GLUT-4 after creatine administration is mediated by the increase in AMP-activated protein kinase (AMPK) activity that occurs as a consequence of decreasing the PCr/creatine ratio.[276] Nevertheless, no direct evidence has been obtained and the mechanisms underlying the increase in GLUT-4 content after creatine supplementation remain to be elucidated.

Low et al.[278] have provided evidence that osmotic swelling of muscle cells is a potent stimulus to muscle glycogen synthesis. In a recent study performed in experimental animals,[279] short-term high-dose creatine feeding enhanced creatine disposal and glycogen storage in skeletal muscle. This occurred irrespective of muscle type and without significant modification of glucose transport rate, muscle GLUT-4 content, circulating insulin levels, glycogen synthase activity, and glycogen synthesis rate. These results support the osmotic swelling theory. An increase in muscle TCr of 30 mmol/kg dm is probably sufficient to induce a degree of cell swelling able to enhance glycogen synthesis.[278,280]

3.5.4 Creatine-Induced Improvement in Muscle Efficiency

Muscle efficiency is the relationship between muscle output and the energy cost for it. In aerobic metabolism, energy cost is measured as $\dot{V}O_2$, whereas muscle output is either speed in running or watts in cycling. Only a limited number of studies have measured $\dot{V}O_2$ during exercise with and without creatine loading.[235,255,256,259,267] Balsom et al.[235] reported a reduction in $\dot{V}O_2$ following the seventh bout of repeated supramaximal cycling exercise using Douglas bag collections of expired air. Stroud et al.[255] found no difference in $\dot{V}O_2$ during submaximal incremental treadmill exercise, and Balsom et al.[256] reported no change in $\dot{V}O_2$ during continuous running at ~ 120% $\dot{V}O_{2max}$ following creatine loading. In both studies, $\dot{V}O_2$ was not analysed breath-by-breath but was averaged over 30-second periods and the exercise bouts were only performed once. Rico-Sanz and Marco[259] reported that the total volume of oxygen consumed by trained cyclists during 3-minute periods of cycling at 90% of the power output at $\dot{V}O_{2max}$ was greater following creatine loading. By contrast, Jones et al.[267] reported an improvement in muscle efficiency after creatine loading (20 g/d for 5 days, and 5 g/d thereafter), since less $\dot{V}O_2$ was required in nine healthy but untrained participants to perform heavy (above the ventilatory threshold) submaximal cycle exercise (6 minutes). In addition, the authors found that the magnitude of the reduction in submaximal $\dot{V}O_2$ with creatine loading was significantly correlated with the percentage of type II fibres in the vastus lateralis muscle ($r = 0.87$; $p < 0.01$).

Individuals in the study by Rico-Sanz and Marco[259] were highly trained long-distance cyclists ($\dot{V}O_{2max}$ ~64 ml/kg/min) whereas participants in the study by Jones et al.[267] were recreationally active in a number of sports but not well trained ($\dot{V}O_{2max}$ ~46 ml/kg/min). It is quite possible, therefore, that there were differences in the proportion of type I fibres in the active muscles between the study participant groups. The addition of creatine to cultures of type II muscle fibres has not been shown to cause an increased respiratory rate.[281] On the other hand, a positive correlation has been reported between the proportion of type I fibres and the increase in the rate of aerobic respiration when creatine was added to a culture of human skeletal muscle fibres.[282] This explains the higher $\dot{V}O_2$ rate at the same muscle output after creatine supplementation in the study by Rico-Sanz and Marco.[259] Therefore, creatine supplementation seems to be able to improve muscle efficiency only when a greater percentage of type II fibres is required (e.g. heavy aerobic exercise above the ventilatory threshold, or when the individuals have a greater percentage of type II fibres). Two mechanisms may explain this:

Excess of H^+ in the Intermembrane Space of Mitochondria

It is well established that after creatine supplementation, an excess of TCr is stored in muscle. This is mainly in the form of creatine (50% of the increase). A plausible hypothesis is that ATP synthesised within the mitochondrial matrix is used by Mi-CK to phosphorylate creatine in the intermembrane mitochondrial space, yielding PCr, ADP and H^+. The ADP liberated by this reaction may be transported back to the matrix where it is rephosphorylated to ATP. Indeed, the liberated H^+ may yield ATP by F1-ATPase (figure 9). Thus, with the same energy cost (the same $\dot{V}O_2$), more ATP may be produced, and, therefore, more muscle output. However, this hypothesis remains to be fully proven.

Shortening of Muscle Relaxation Time

Compared with sustained muscle contractions, the excess energy expenditure during no-sustained muscle contractions (e.g. running, cycling) is, at least, partly accounted for by the energy cost of muscle relaxation.[283] Thus, during high-intensity cyclic joint movements, facilitation of muscle relaxation may contribute to reduce total energy expenditure, both by diminishing the energy cost of relaxations and by reducing co-contraction activity.[284] Wakatsuki et al.[285] found that creatine feeding in rats raised muscle PCr and shortened muscle relaxation time. Accordingly, PCr depletion induced by β-guanidinopropionic acid feeding increased muscle relaxation time. Van Leemputte et al.[284] found that, in humans, creatine supplementation (20g/d, for 5 days) reduced, consistently

by ~20%, the muscle relaxation time from the first to the last of 12 maximal isometric elbow flexions interspersed, without affecting torque production or electromyogram activity. Individuals with low initial muscle creatine levels exhibited a greater increment of muscle creatine store on creatine intake which, in turn, may result in more pronounced shortening of muscle relaxation time; this also occurs in individuals with slow initial muscle relaxation rates.[284]

The major determinants of human skeletal muscle relaxation time are the rate of cross-bridge detachment and the decay of cytoplasmic Ca^{2+} by sarcoplasmic Ca^{2+}-ATPase activity.[286,287] After creatine loading, muscle PCr increases and CK serves to optimise free energy of ATP hydrolysis by maintaining a low concentration of free ADP, which is very critical to Ca^{2+}-ATPase pump efficacy.[23] Therefore, the rise in local PCr concentration may allow sarcoplasmic Ca^{2+}-ATPase to operate at a higher thermodynamic efficiency and thereby facilitate muscle relaxation.[284,288] Because sarcoplasmic Ca^{2+}-ATPase is much more sensitive than myosin ATPase to a decrease in the free energy of ATP hydrolysis,[289] creatine loading may facilitate muscle relaxation without simultaneously affecting force production during contraction.[284] Further research is necessary to clarify this topic.

In summary, a creatine-induced improvement in aerobic performance mainly in individuals with high type II fibre content seems probable. Therefore, creatine may have more benefit than simply enhancing high-intensity exercise performance. Moreover, most endurance athletes perform intermittent high-intensity exercise and/or incorporate intermittent sprinting training (e.g. runners, cyclists), so they may improve quality of training with creatine supplementation. In running, it is possible that any advantage from the creatine loading might be negated by the concurrent increase in body mass which would tend to increase the energy cost of exercise. In cycling, where body mass is less important to performance at least on the flat, it is feasible that creatine loading could enhance endurance-exercise performance. Additional studies are needed to clarify the mechanism by which $\dot{V}O_2$ during heavy exercise is reduced following creatine loading, mainly in individuals with high rates of type II muscle fibres and in non-weightbearing endurance activities.

3.6 Weight Loss and Strength Improvement

In weight-class sport events, weight loss before weight-in and its immediate regain thereafter may be important (Gutiérrez A et al., unpublished observations).[290] In this way, anaerobic performance may decrease with energy restriction practised for intentionally rapid weight loss. Rockwell et al.[291] have reported that creatine supplementation (20 g/d, for 4 days) during energy restriction (18 kcal/kg/d, for 4 days) reduced bodyweight by 2.2 ± 0.4kg (2.7%) compared with the reduction observed in the placebo group (2.8 ± 0.2kg [3.6%]). In addition, the reduction in fat free mass was higher in the placebo (2.4 ± 0.3%) than in the creatine group (1.4 ± 0.4%). In this group, muscle creatine and PCr content increased by 15–16%, so total performed work during the sprints, expressed per bodyweight, tended to be 3.8% higher in the creatine group and 0.5% less in the placebo group. In another study,[292] creatine supplementation during energy restriction to induce weight loss, maintained peak torque and work at peak torque, whereas weight loss without creatine supplements resulted in decrements in peak torque during maximal knee-extensor exercise. These results suggest that anaerobic performance may be enhanced during short-term energy restriction in athletes who supplement with creatine.

4. Safety and Adverse Effects

This section provides an update regarding the adverse effects of creatine supplementation as an ergogenic aid based on results reported in the literature.

4.1 Body Mass Increase

It has often been reported that short-term creatine ingestion is accompanied by a 1–2% increase in body mass.[9,131,137,293-296] In this regard, short-term supplementation (20g creatine per day, for 10 days) is as effective as longer periods (>10 days).[297] Such body mass increments are caused

by an increase in fat-free mass. Indeed, several studies indicated that creatine loading increases fat-free mass.[125,180,298-300] The body mass increment after creatine supplementation is lower in women than in men.[300,301] In elderly people (range 60–80 years of age) the body mass either did not increase significantly, or was less than 1kg, after creatine supplementation.[185,186,302-305] This increase in bodyweight in the elderly was also mainly accounted for by an increase in fat-free mass.[303]

In summary, creatine supplementation during the short- or medium-term, increases body mass mainly by an increase in the fat-free mass.

4.2 Water Retention

The previous studies demonstrated gains of 1kg or greater after creatine loading, and researchers arbitrarily suggested water retention as a possible causative factor, yet few researchers have addressed this issue directly. Hultman et al.[122] suggested that this increase in bodyweight during short-term creatine supplementation is likely to be attributable to body water retention, since they observed a 0.6L decline in urinary volume after creatine ingestion (20 g/d, for 6 days). In this way, the increase in body mass could be partially (55%) attributed to an increase in the body water content, particularly in the intracellular compartment.[215,306-308] The recent work of Ziegenfuss et al.[306] indicated that both a body mass gain and an expansion of total body water occurred with 3 days of creatine supplementation. Using multifrequency bioimpedance analysis, the investigators noted that the increase in body water was specific for the intracellular volume of the muscle. The authors suggested that water retention was related to the osmotic load triggered by the enhanced cellular uptake of creatine. Kern et al.,[308] using bioelectrical impedance, found greater gains in total body water and bodyweight in the creatine group compared with the placebo. One recent study[309] showed increases in fat-free mass with no effect on total body water. Additional studies will clarify whether creatine loading may increase total body water.

4.3 Muscle Cramps

There have been a number of reports of muscle cramps in athletes receiving creatine (for review see references[310,311]). In the reviews of Juhn and Tarnopolsky,[310] and Schnirring,[311] the authors reported unsubstantiated anecdotal reports of cramping. It has been reported that creatine supplementation (0.3 g/kg body mass/d for 6 days, followed 0.03 g/kg body mass/d for 28 days) increased anterior compartment pressure in the lower leg at rest and following 20 minutes of running at 80% of maximal aerobic power.[312] Moreover, anecdotal reports from athletes have claimed that creatine supplementation may induce muscle cramps, but there is no evidence that these cramps are directly related to the creatine ingestion. In fact, these cramps might be caused by the intensity of exercise or the imbalance in muscle electrolytes, rather than to creatine ingestion. Consequently, athletes should consume adequate water and electrolytes, as these are likely to be the most common cause of muscle cramps.

Based on the possibility that creatine supplementation may result in an increase in fluid retention and total body water (see section 4.2), creatine may provide thermal regulatory benefits during exercise in the heat. In this regard, augmented repeated sprint cycle performance has been reported in the heat without alterations in the thermoregulatory response after creatine supplementation.[313] Additionally, Kern et al.[308] reported that the rise in core temperature during the cycle ride was attenuated by creatine supplementation in comparison to placebo consumption. Gains in total body water were related to the attenuation of temperature rise during the ride following supplementation ($n = 19$, $r = 0.569$, $p = 0.011$). Indeed, during post-supplementation testing, the temperature rise of the creatine group averaged 0.37°C lower than pre-supplementation over 60 minutes of exercise at 37°C. Further, amid anecdotal reports that creatine causes dehydration, muscle cramping and heat exhaustion, the participants in this study reported no overt adverse effects of creatine supplementation. In fact heart rate, hematocrit, body water loss and temperature rise were clearly not adversely affected by creatine supplementation as

would be expected if these testimonial reports were true. On the other hand, there is no evidence from any study indicating that creatine loading increases the incidence of dehydration/thermal stress,[308,313-316] cramping[309,313,317-325] and electrolyte status.[130,215,306,309,313,326] In addition, there are numerous studies that did not report any kind of trauma/injury or muscle impairment after creatine supplementation.[124,301,313,317-320,322,323,325,327,328]

In summary, there is no direct evidence that oral creatine supplementation is responsible for dehydration/thermal stress or muscle impairment. Further research will clarify whether creatine loading may be beneficial with regard to performing exercise in the heat.

4.4 Gastrointestinal Complaints

When creatine is ingested, some discomfort can occur if creatine is incompletely dissolved before ingestion.[297] Additionally, a survey by Juhn et al.[329] reported that when creatine is ingested in large doses (40 g/d) or during long-term periods (3–5 months), nausea, vomiting or diarrhoea can be produced after creatine ingestion. Since the Juhn et al.[329] survey had no control group, it is unclear whether the adverse effects reported had any relationship to creatine or were influenced by 'anecdotal side effects' reported in the media. In fact, recent well-controlled studies reported no effect of creatine supplementation on gastrointestinal disturbance,[130,131,137,234,301,317-320,322,323,325,327,328] and several of these papers reported a lower incidence when compared with those taking placebos. It is also possible that some gastrointestinal complaints can be caused by co-ingestion of other substances or a deficient quality of creatine supplement. In these cases, gastrointestinal transit and absorptive capacity of the gut can be altered. One anecdotal report[330] suggested that creatine consumed during or immediately before physical exercise may be problematic, but further research is needed to clarify this.

4.5 Renal Function

There is little scientific evidence suggesting that creatine causes impairment of renal function. Indeed, there are only four case reports[331-334] relating renal problems to creatine supplementation. Kuehl et al.[331] reported renal insufficiency induced by creatine supplementation (10 g/d, for 3 months) in a 19-year-old Division I football player. Pritchard and Kalra[332] reported negative effects after long-term creatine ingestion (15 g/d for 7 days, followed by 2 g/d for 49 days) in an individual with pre-existing renal disease, but renal function was re-established after stopping creatine ingestion. Koshy et al.[333] found one case of interstitial nephritis in a patient who had taken 20g of creatine daily for 4 weeks. After stopping the creatine supplements, his renal function became normal. Loud et al.[334] found an impairment in renal function after creatine loading. In this study, the study participant ingested liquid creatine (with only 25mg of creatine per serving). In these case reports, elevations in serum Crn (e.g. 1.5–1.7 mg/dl) were initially used to diagnose renal stress. In addition, no controls were provided for comparison. Therefore, these case reports of renal problems may be misleading. Others studies[297,335,336] have criticised the conclusions drawn from these case studies. Studies that have evaluated renal function in response to creatine supplementation using urinary Crn clearance[314,337-341] and/or using clearance of a nonradioactive iodinated contrast technique (to assess glomerular filtration)[129] reported no renal problems in individuals receiving creatine for up to 5 years (retrospective analysis) or 21 months (monitored supplementation).

In summary, there is no scientific evidence that creatine supplementation at the usual dosages is able to induce any impairment in renal function. In any case, renal function must be adequately monitored in individuals taking large amounts of creatine and, in general, the creatine supplement must be used with precaution in individuals at risk of presenting with renal dysfunction.

4.6 Liver Function

Earnest et al.[342] did not show any significant increase in serum urea and bilirubin throughout the duration of a creatine supplementation (20 g/d for 5 days, followed 10 g/d for 51 days) programme. The same group[343] investigated the levels of serum liver enzymes and did not observe significant

changes during the 8 weeks of creatine supplementation. Robinson et al.[124] did not report adverse effects on hepatic function after short-term (20 g/d, for 5 days) or long-term (3 g/d, for 63 days) creatine supplementation. These findings were confirmed by additional references showing no impact on muscle and liver enzymes following creatine supplementation, even after long-term administration.[341,344-346] In contrast, other investigators reported significant increases in aspartate aminotransferase and alanine aminotransferase plasma levels after creatine supplementation (15.75 g/d, for 28 days),[130] but the elevated activities of the marker enzymes were most likely caused by exercise-induced muscle response.

In summary, in healthy individuals, oral creatine supplementation at the correct dose does not induce significant changes in liver function tests. Nevertheless, it seems reasonable to monitor liver function in individuals receiving creatine supplementation.

4.7 Creatine as a Precursor of Mutagens and Carcinogens of the Amino-Imidazo-Azaarene (AIA) Class

Recent opinion statements have suggested that creatine may add to human carcinogenesis by forming either amino-imidazo-azaarene (AIA) mutagens or formaldehyde that covalently modify guanine bases and thereby result in DNA mutations. This section analyses these assumptions.

4.7.1 Creatine-Induced Formaldehyde Formation

It has been suggested[347] that chronic administration of large quantities of creatine may be metabolised to methylamine. Methylamine may be transformed to formaldehyde and hydrogen peroxide by semicarbazide-sensitive amine oxidase (SSAO)[348] (figure 18). Formaldehyde has the potential to cross-link proteins and DNA, leading to cytotoxicity,[349,350] and carcinogenic effects in gastrointestinal tract mucosa cells.[351] This mechanism has also been related to vascular injuries, diabetes mellitus and renal failure.[348] These findings concerning the carcinogenic role of formaldehyde have prompted statements[347,348] assuming a putative carcinogenic role of creatine supplementation. Nevertheless, there is little, if any, evidence

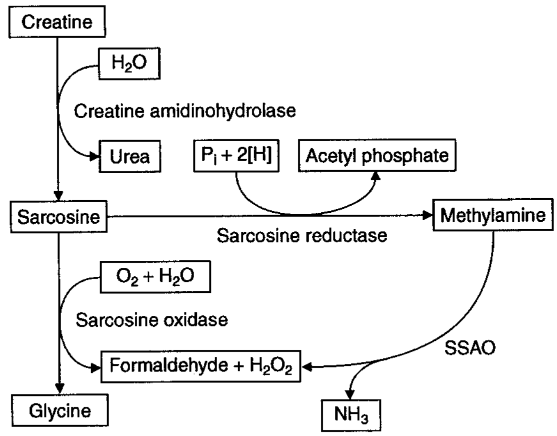

Fig. 18. Theoretical formation of formaldehyde. Methylamine is transformed to formaldehyde and hydrogen peroxide (H_2O_2) by semicarbazide-sensitive amine oxidase (SSAO). Indeed, there is no evidence that creatine increases methylamine or formaldehyde. P_i = inorganic phosphate.

that creatine increases methylamine or formaldehyde. In addition, there are no studies even suggesting an increased risk of cancer with creatine. In contrast, there are several studies[18,19,352-356] showing an anticarcinogenic effect of creatine and its analogues. Therefore, there is no evidence at present that creatine increases carcinogenesis.

4.7.2 Creatine-Induced AIA Mutagens

The discovery in 1977 that cooked fish and beef showed highly mutagenic activity[357,358] began an intensive search for the mutagens present in these foods. A number of studies showed that these mutagens were formed during the pyrolysis of amino acids and proteins, and during the cooking of a variety of muscle meats.[357-361] The novel mutagens were identified as heterocyclic amines. The major subclass of heterocyclic amines found in cooked meats was identified as the AIAs. Sources of AIA mutagens were broiled or fried fish, cooked chicken, beef and pork meat, fried eggs, as well as fumes from cooking meat.[8] A large body of evidence indicates that creatine and/or Crn are important precursors of AIA mutagens:

- In different fried bovine tissues as well as in meat extracts, beef-flavour bouillons, and gravies, mutagenicity correlated with the creatine and/or Crn content of the sample.[362,363]

- Addition of creatine or PCr to meat samples or to beef extracts before the cooking process increased mutagenicity up to 40-fold and AIA contents up to 9-fold.[362,364] Conversely, treatment of beef meat with creatinase before frying reduced the creatine content and the mutagenicity by 65 and 73%, respectively.[365]
- Artificial model systems have widely been used to study both the probable precursors and the reaction pathways leading to the formation of AIA mutagens. In these models, creatine or Crn was mixed with a variety of substances and incubated for different periods of time at temperatures of 100–250°C. Omission of creatine or Crn from these model systems greatly reduced mutagenicity.[366]

Taken together, these findings suggest a mutagenic effect of creatine only when this is cooked at high temperatures. Since the formation of AIA mutagens depends largely on cooking temperature and duration,[360,367,368] it is improbable that AIA mutagens may originate following creatine supplementation. In fact, there is no scientific evidence of AIA formation after creatine loading.

In summary, there is no scientific evidence that creatine supplementation may add to human carcinogenesis by forming either AIA mutagens or formaldehyde. In the numerous short- and long-term studies[124,128,164,186,202,297,346,369-371] reporting general markers of clinical health/safety, none has shown any adverse effect when quality creatine supplements are used. It is also interesting to note that recent studies in infants between the ages of 2–4 years with a genetic disturbance in creatine synthesis have shown remarkable clinical, biochemical and functional improvements following creatine supplementation, in doses ranging from 350–500 mg/kg bodyweight (136.4–227.3 mg/lb bodyweight) that were maintained for over 25 months.[372-377] This dose is up to 1.67 times the recommended loading dosage. No adverse effects were reported, including no aggravation of seizures in one infant who presented with intractable seizures (including rare grand mal seizures) before being treated with creatine.[377]

4.8 Impurity of Creatine Supplements

Data on the impurity of commercially available creatine supplements are frightening. Creatine is obtained from natural sources (sarcosine) present in animal muscles. During the industrial production of creatine monohydrate, sarcosine and cyanamide are used. Consequently, variable amounts of contaminants (dicyandiamide, dihydrotriazines, Crn, ions) are generated and, thus, their tolerable concentrations must be defined and made available to the consumer. This impurity of creatine supplements may be the origin of some of the anecdotal adverse effects following creatine supplementation.

5. Conclusion

Creatine is a legal substance consumed by amateur and professional athletes as an ergogenic aid, being the most widespread, effective and safe ergogenic aid in the world. Its use is extended to trained and untrained individuals, men and women, adolescent and elderly individuals. Creatine supplementation has been advocated to improve high-intensity exercise, intermittent high-intensity exercise, and even endurance exercise (mainly in non-weight-bearing endurance activities). There is no scientific evidence of any adverse effect following creatine supplementation in healthy individuals, even during long-term administration. Nevertheless, renal and liver monitoring must be performed in individuals with previous pathology in these organs. In this paper, the ergogenic use (including adverse effects) of creatine has been reviewed. Additional studies should be performed to clarify this usage (including metabolism, pharmacokinetics and adverse effects).

Acknowledgements

We thank Dr Conrad Earnest for his assistance in reviewing the manuscript and the provision of highly valuable information. This paper has been prepared without any external funding. No present or past conflict of interest exists for any of the authors or their institutions.

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Correspondence and offprints: *Manuel J. Castillo Garzón*, Department of Physiology, School of Medicine, University of Granada, E-18071 Granada, Spain.
E-mail: mcgarzon@ugr.es

Nitric Oxide 22 (2010) 110–119

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Review

Nutritional epidemiology in the context of nitric oxide biology: A risk–benefit evaluation for dietary nitrite and nitrate

Andrew Milkowski[a], Harsha K. Garg[b], James R. Coughlin[c], Nathan S. Bryan[b,d,*]

[a] Muscle Biology Laboratory, Department of Animal Sciences, University of Wisconsin, Madison, WI, USA
[b] Brown Foundation Institute of Molecular Medicine, The University of Texas Health Science Center, Houston, TX, USA
[c] Coughlin & Associates, Laguna Niguel, CA, USA
[d] The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, TX, USA

ARTICLE INFO

Article history:
Received 6 August 2009
Revised 25 August 2009
Available online 11 September 2009

Keywords:
Diet
Nitrite
Nitrate
Vegetables
Cured meats
Cancer
Cardiovascular disease

ABSTRACT

The discovery of the nitric oxide (NO) pathway in the 1980s represented a critical advance in understanding cardiovascular disease, and today a number of human diseases are characterized by NO insufficiency. In the interim, recent biomedical research has demonstrated that NO can be modulated by the diet independent of its enzymatic synthesis from L-arginine, e.g., the consumption of nitrite- and nitrate-rich foods such as fruits, leafy vegetables, and cured meats along with antioxidants. Regular intake of nitrate-containing food such as green leafy vegetables may ensure that blood and tissue levels of nitrite and NO pools are maintained at a level sufficient to compensate for any disturbances in endogenous NO synthesis. However, some in the public perceive that dietary sources of nitrite and nitrate are harmful, and some epidemiological studies reveal a weak association between foods that contain nitrite and nitrate, namely cured and processed meats, and cancer. This paradigm needs revisiting in the face of undisputed health benefits of nitrite- and nitrate-enriched diets. This review will address and interpret the epidemiological data and discuss the risk–benefit evaluation of dietary nitrite and nitrate in the context of nitric oxide biology. The weak and inconclusive data on the cancer risk of nitrite, nitrate and processed meats are far outweighed by the health benefits of restoring NO homeostasis via dietary nitrite and nitrate. This risk/benefit balance should be a strong consideration before there are any suggestions for new regulatory or public health guidelines for dietary nitrite and nitrate exposures.

Introduction

Today, we are bombarded with media reports of studies relating diet to a number of chronic diseases, including coronary heart disease, cancer, type 2 diabetes and osteoporosis. For the past several decades, observational studies of diet and cancer have yielded many inconsistent results [1,2]. Given the limited variation in dietary intakes within many study populations and the seemingly weak diet–cancer associations that have been observed, results of such studies depend critically on an accurate assessment of dietary exposure [3,4]. Measurement error in exposure can lead to serious errors in the reported relative risks of cancer for dietary intakes and can substantially reduce the statistical power to detect true existing relationships [3,4]. Extreme caution is required when interpreting associations, or the lack thereof, between dietary factors and disease.

Overall, the media does a fairly good job of reporting the limitations of the studies and the fact that the published results are merely expressions of risk probability. However, a recent, prominent *Perspective* article in the New England Journal of Medicine [5] noted that all too frequently, what is conveyed about health and disease by many journalists is wrong or misleading, especially when they ignore complexities or fail to provide context. When this happens, the public health messages conveyed are inevitably distorted or inadequate. Therefore, the news media need to become more knowledgeable and to more fully embrace their role in delivering accurate, complete and balanced messages about health.

This is especially needed when the results being communicated to the public are diet-health associations, due mainly to the complexity of the diet compared to a drug/placebo clinical trial. There has been a dramatically increased interest in nutrition and health over the past decade. What we eat or don't eat is constantly being linked to various diseases, and there is a constant flow of anxiety-provoking media headlines on television, radio, print and more recently the Internet. "Carcinogen-of-the-month" reporting has become very alarming to consumers, and dietary epidemiological

* Corresponding author. Address: Brown Foundation Institute of Molecular Medicine, The University of Texas Houston Health Science Center, 1825 Pressler St., SRB 530B, Houston, TX 77030, USA. Fax: +1 713 500 2447.
E-mail address: Nathan.bryan@uth.tmc.edu (N.S. Bryan).

1089-8603/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.niox.2009.08.004

studies always seem to be contradicting each other, leading to much nutrition nonsense and food faddism. Because of these fears, consumers become sporadic or chronic avoiders of specific foods and ingredients, such as salt, fat, soft drinks, artificial sweeteners, carbohydrates in general, coffee/caffeine and meat products.

In a recent issue of Archives of Internal Medicine, Sinha et al. [6] reported in a large prospective study that red and processed meat intakes were associated with modest increases in total mortality, cancer mortality, and cardiovascular disease mortality. Like many other studies, it failed to completely consider several additional factors that can contribute to chronic disease, including participants' behavior as to alcohol and tobacco use, exercise, weight and access to health care. It also failed to recognize the role of beef, pork and other red meat in providing essential and under-consumed nutrients. In response to such reporting deficiencies, many individuals become either confused or alarmed about their own personal situation. There are also calls by numerous public health and consumer organizations to change our lifestyles as a matter of public policy. Therefore, it is important for participants in the social debate to also understand the strengths and limits of epidemiological research. We will review the science of epidemiology, introduce the criteria for interpretation of the data and then discuss some published reports on diet and risk of disease. Since foods are heterogenous and complex in terms of their composition and contribution to the overall diet, ascribing an individual component of food as "good" or "bad" is fraught with difficulty. We will present a cursory review of current epidemiological data, but we will also focus primarily on the context of nitrite and nitrate in foods and what is reported about them, in order to present a balanced view of dietary sources of essential nutrients and a potential risk–benefit evaluation.

Epidemiology as a scientific discipline

Modern epidemiology is the branch of medicine that deals with the study of the causes, distribution and control of disease frequency in human populations. Historically, epidemiology began as the study of epidemics of infectious disease. Epidemiology essentially looks for patterns of disease (time, place, exposures, personal characteristics). Nothing affects our health more than what we choose to eat. Many studies relate the association between processed meats and cancer to their nitrite and nitrate contents. The primary concern for exposure to dietary nitrite (and nitrate as a precursor to nitrite) is its propensity to form potentially carcinogenic *N*-nitrosamines and their consequent potential to cause human cancer [7,8]. In terms of human cancer risk and diet, most of the publications, discussion and media attention have focused on data from epidemiological studies. Therefore, it is important to understand the different types of dietary epidemiological studies, while at the same time noting that epidemiological associations reported between dietary components, specific foods (or food groups) and chronic disease are rarely sufficient to establish cause and effect relationships. The results of epidemiological investigations must also be evaluated through other types of supportive studies (animal studies, mechanistic studies, metabolic studies, human clinical intervention trials, etc.) before persuasive causal relationships can be firmly established [9,10].

There are several types of epidemiological studies, each with their own strengths and weaknesses [10]. Ecologic/descriptive studies are the simplest and least persuasive type. They characterize differences between large and diverse populations by simple generalizations and can help formulate hypotheses; however, they cannot control for potential confounding factors, i.e., factors that are known risk factors for the disease. Case-control studies focus on individuals and provide stronger evidence for an association than ecologic studies. "Recalled" past diets of individuals diagnosed with a disease (cases) are compared to those of individuals without the disease (controls) in a retrospective case-control study. Many researchers rely on this type of study because of lower cost, smaller sample size and ability to study many potential factors. However, such retrospective studies are subject to recall bias and unavailable or incomplete data particularly accurate dietary exposure data. There may also be questions regarding adequacy of the "control" group. Follow-up (prospective cohort) studies, on the other hand, are considered to provide the most definitive information and are the most persuasive study design. In these studies a cohort (group) of individuals, who do not yet have a specific disease, are selected and followed over a period of time while collecting specific information regarding diet and other factors related to the development of the disease. However, prospective studies are more costly and require more time and larger numbers of subjects. Following on the results of these types of epidemiological study, it is critical to conduct human, randomized clinical intervention studies in order to ultimately establish cause–effect relationships.

Interpretation of epidemiological studies demands causation criteria

To evaluate research findings in any area of scientific investigation, certain scientific standards, established by experts in each field, need to be applied. This is especially true when trying to determine the health effects of the inclusion or exclusion or varying levels of components in the diets of humans. The eminent British biostatistician and epidemiologist A. Bradford Hill published a seminal paper in 1965 [11] offering a number of interpretation criteria that would be useful when interpreting the statistical results observed in epidemiological studies. The goal of these criteria was to guide epidemiologists in inferring causation (or establishing "causal inference") from the associations observed in such studies. In effect, Bradford Hill tried to provide a framework to judge whether associations observed in a body of epidemiological work could be determined to be causal. Since the time of his publication, these criteria (which Bradford Hill urged others to call "viewpoints" and not true criteria) have become a de facto standard to evaluate the statistical associations found in epidemiological research.

Bradford Hill pointed out, however, that none of these nine viewpoints provide indisputable evidence for or against a cause and effect hypothesis. What he claimed they could do, with greater or less strength, was to help answer the fundamental question of whether there is any other way of explaining the set of observed facts, i.e., is there any other answer equally, or more likely than cause and effect? His criteria are still very relevant to the scientific and public discussion of diet and disease relationships. Bradford Hill's causation criteria are described below, with examples given to help illustrate each of the criteria.

The interpretation criteria for epidemiological associations presented by A. Bradford Hill in 1965.

1. Strength of association	Magnitude of the effect
2. Temporality	Exposure must precede the disease
3. Consistency	Similar findings in many studies without contradictory results
4. Theoretical plausibility	Biological/Physiological/Metabolic knowledge supports the findings
5. Coherence	No alternate hypotheses
6. Specificity	No confounding factors are found
7. Dose–response	Higher risk with higher exposure
8. Experimental evidence	Animal and *in vitro* experiments show the effect
9. Analogy	Similar findings in other situations can apply

1. *Strength of association:* The stronger the relationship between the independent variable (the risk factor) and the dependent variable (the disease), the less likely it is that the relationship is due to an extraneous variable (a confounder). For example, the relative risk of smokers developing lung cancer is around 10 or above (i.e., a 10-fold higher risk than for non-smokers). The relative risk associated with consumption of grains containing mycotoxins and liver cancer is around 6. These are quite strong associations, and by contrast, modest relative risks on the order of 2 or less should be viewed with skepticism due to the likelihood of many potential confounders. This was recognized in 1994 when the U.S. National Cancer Institute publicly indicated that relative risks values less than 2 were not strong enough to use for public policy pronouncements [12] (this document is available through the National Cancer Institute's CancerFax and CancerNet services, and in the News Section of the NCI's PDQ database. To get the document from CancerFax, dial +1 301 402 5874 from the handset on your fax machine and follow the recorded instructions to receive the contents list: U.S. National Cancer Institute Press Release, Oct 16, 1994).

2. *Temporality:* The exposure must precede the disease by a reasonable amount of time, i.e., a cause must precede an effect in time. For example, cigarette smoking over a period of years is well-established to increase lung cancer risk. While this relationship seems obvious, there have been published findings that have occasionally violated this criterion.

3. *Consistency:* Multiple observations of an association, with different people under different circumstances and with different measurement instruments, increase the credibility of a causal finding. Different methods (e.g., ecological, cohort and case-control studies) should produce the same conclusion. The relationship should also hold for different groups of people (in males and females, in different populations on different continents). This criterion is greatly debated, however, because consistency is often in the eye of the beholder. Some reviewers can conclude that six of ten studies with a statistically significant association represent a consistent finding, while others can see the same set of data as inconsistent.

4. *Theoretical plausibility:* It is easier to accept an association as causal when there is a rational and theoretical basis for such a conclusion supported by known biological and other facts. While our knowledge of physiology today is vast, there is still much that is unknown about the complex interactions of ingested foods and food components and their metabolism, interactions and potential adverse effects.

5. *Coherence:* A cause-and-effect interpretation for an observed association is clearest when it does not conflict with what is known about the variables under study and when there are no plausible competing theories or rival hypotheses. In other words, the association must be coherent with other existing knowledge. The conclusion that smoking causes lung cancer, based on decades of epidemiologic, laboratory animal, pharmacokinetic, clinical and other biological data, showed that all available facts stuck together as a coherent whole.

6. *Specificity in the causes:* In the ideal situation, the effect has only one cause. In other words, showing that a disease outcome is best predicted by one primary factor adds credibility to a causal claim. But this is often not the norm. High consumption of one food, food ingredient or nutrient can be covariate with low consumption of another food or ingredient, and it is often difficult to determine which of the two is more important. Other non-dietary confounders must also be considered. For example, chronic *Helicobacter pylori* infection is a strongly suspected risk factor for gastric cancer [13]. Nutritional epidemiological studies that fail to account for the incidence of this infection in the population studied should therefore be viewed with caution.

7. *Dose–response relationship:* There should be a direct biological gradient (or dose–response) between the risk factor (the independent variable) and people's status on the disease variable (the dependent variable). Many dietary epidemiological studies report a significant statistical trend for dose–response, but on closer examination, the data can be highly inconsistent. The data may only span a very narrow range of dietary intakes or in some cases such a wide range of intakes that upper intakes are representative of a grossly unbalanced diet. Calculated relative risks may sometimes decline to non-significant levels with higher intakes, or in some cases one quartile of intake may have a high enough calculated risk to skew the overall results. Such data patterns, when deviating from the linear dose–response, need to be considered as unlikely dose–response relationships and also be considered as part of the consistency criterion.

8. *Experimental evidence:* Any related research (animal, *in vitro*, etc.) that is based on experiments and supports the conclusions of epidemiological studies will make a causal inference more plausible. This must be tempered with the understanding that animals are not people, and results from lifetime, chronic feeding bioassays, where rodents are dosed at levels up to and sometimes exceeding maximum tolerated doses (MTDs), in such testing programs as the U.S. National Toxicology Program, may often be very useful but are not definitive by themselves.

9. *Analogy:* Sometimes a commonly accepted phenomenon in one area can be applied to another area. For instance, a newly discovered *N*-nitrosamine may be considered to be a carcinogen if it has structural similarities to other well-known carcinogenic *N*-nitrosamines. However, analogy is an obtuse criterion, and thus is considered to be a weaker form of evidence. This makes the application of the analogy consideration even more uncertain than the application of considerations on plausibility and coherence.

Bradford Hill developed his list of "criteria" that continues to be used today. When using them, it's important not to forget Hill's own advice: "None of these nine viewpoints can bring indisputable evidence for or against a cause and effect hypothesis... What they can do, with greater or less strength, is to help answer the fundamental question – is there any other way of explaining the set of facts before us, is there any other answer equally, or more, likely than cause and effect?" Phillips and Goodman [14] have recently provided observations on the use of Hill's "causal criteria." They argued that the uncritical repetition of Hill's criteria is probably counterproductive in promoting sophisticated understanding of causal inference, but they set out a simplified list of the key Hill considerations that they thought is worthy of repeating:

- "Statistical significance should not be mistaken for evidence of a substantial association.
- Association does not prove causation (other evidence must be considered).
- Precision should not be mistaken for validity (non-random errors exist).
- Evidence (or belief) that there is a causal relationship is not sufficient to suggest action should be taken.
- Uncertainty about whether there is a causal relationship (or even an association) is not sufficient to suggest action should not be taken."

The authors pointed out that while these points may seem obvious when stated so briefly and bluntly, causal inference and health policy decision-making would benefit tremendously if they were considered more carefully and more often.

The International Food Information Council (IFIC) Foundation has addressed the issue of "How to Understand and Interpret Food and Health-Related Scientific Studies" [15]. They discuss the types

of epidemiological research studies and what journalists, educators and health professionals should look for when critically reviewing scientific studies. When evaluating an epidemiological report, IFIC suggested that the following questions can be asked:

- Could the study be interpreted to say something else?
- Are there any methodological flaws in the study that should be considered when making conclusions?
- Are the study's results generalizable to other groups?
- How does the work fit with the body of research on the subject?
- What are the inherent limitations of this type of study and does the research design fit the stated purpose of the study?
- Has the author omitted important points in the background section which could have a meaningful effect on the study design or interpretation of the results?
- Are there any major design flaws in the study and are the data collection measures appropriate to answer the study questions?
- Were methodological limitations acknowledged and discussed and what influence might these have had on the results?
- What is the real and statistical significance of the results?
- To whom do the results apply and how do the results compare to those of other studies on the subject?
- Are the conclusions supported by the data?
- Are the conclusions of the study related to the stated purpose of the study? If not, does the study design and results support the secondary conclusions?

A recent Commentary addressed the important issue of false-positive results that are inherent in the testing of hypotheses concerning cancer and other human illnesses [16]. The authors point out that epidemiology has been increasingly criticized for producing results that are often sensationalized in the media and sometimes fail to be upheld in subsequent studies. They suggested general guidelines or principles, including editorial policies requiring the prominent listing of study caveats, which may help reduce the reporting of misleading results. The authors also urged increased humility by study authors regarding findings and conclusions in epidemiology, noting that this would go a long way toward diminishing the detrimental effects of false-positive results in several areas: (1) the allocation of limited research resources; (2) the advancement of knowledge of the causes and prevention of cancer and (3) the scientific reputation of epidemiology. They concluded that such efforts would help to prevent oversimplified interpretations of results by the media and the public.

Diet and cardiovascular disease

Now with these epidemiological criteria in mind, we will present the available data on certain foods and risk of specific diseases, namely cardiovascular disease (CVD) and cancer. Nothing is more important to our health than our diet, and there are well-established and recognized dietary patterns that confer health benefits. Numerous dietary epidemiological data have generally indicated an inverse relationship between dietary intake of fruits and vegetables and incidence of both CVD and cancer. The specific constituents of fruits and vegetables that afford protection from CVD have and continue to be widely researched and debated. Although there are many dietary components which may have protective effects, antioxidants appear to be one of the groups of phytochemicals that play a significant role.

One group of antioxidants present in fruits and vegetables is known as "polyphenols" or "polyphenolics" and is believed to neutralize free radicals formed in the body, thus minimizing or preventing damage to cell membranes and other cell structures. Although antioxidants are generally credited with improving states of oxidative stress, epidemiological studies which have evaluated the efficacy of supplementation with a high dose of an antioxidant alone, such as vitamin E, have shown no apparent improvement and in some cases a decrease in cardiovascular protection [17]. Yet, when these antioxidants are consumed through the diet in the form of fruits and vegetables, there is a significant degree of protection, best represented by the decrease in mortality rates from CVD of those consuming the popular Mediterranean diet [18]. Although Mediterranean diets vary by country, seasonal availability of ingredients and traditional cooking habits, they all tend to be rich in fruits, vegetables and monounsaturated fatty acids while low in saturated fat and meat. Epidemiological studies, such as the Seven Countries Study [19–25], have led to theories describing why and how the Mediterranean diet may promote a lower incidence of CVD, and antioxidants are emerging as a key component. However, since antioxidant supplementation alone does not confer the cardiovascular benefits, there must be more to explain the cardioprotection afforded by fruits and vegetables.

The data and emerging story presented in this context provide a convincing argument for nitrite and nitrate as probable protective components [26,27]. The Seven Countries Study constitutes the first nutritional epidemiological investigation that provided solid data for cardiovascular disease rates in different populations. The Seven Countries Study is the prototypical comparison study of populations, made across a wide range of diet, risk, and disease experience. It was the first to explore associations among diet, risk, and disease in contrasting populations (ecologic correlations). Central chemical analysis of foods consumed among randomly selected families in each area, plus diet-recall measures in all the men, allowed an effective test of the dietary hypothesis. The study was unique for its time, in standardization of measurements of diet, risk factors, and disease; training its survey teams; and central, blindfold coding and analysis of data. In this study the results for all-cause death rates in Greece, Japan and Italy were quite favorable compared with the USA, Finland, the Netherlands and the former Yugoslavia; the results also showed a lower incidence of CVD after a 5-year follow-up for the same countries that exhibited low mortality. The diet consumed by the Mediterranean cohorts studied was associated with a very low incidence of CVD and was called the *Mediterranean diet* by Keys [28]. Following the Seven Countries Study, the Mediterranean diet has oftentimes been singled out as a healthy diet. Additional studies later confirmed the association of the Mediterranean diet with decreased incidence and prevalence of chronic diseases, mainly CVD, in countries where it was consumed.

There are food peculiarities for the different populations in the Mediterranean basin. However, beyond the apparent differences, there are nutritional characteristics common to all or most of the diets in the Mediterranean region. The Mediterranean diet is characteristically low in saturated and high in monounsaturated fats (olive oil), low in animal protein, rich in carbohydrates, and rich in vegetables and leguminous fiber. People consuming a Mediterranean diet eat a relatively large amount of fish and white meat, abundant fruits and vegetables and a low amount of red meat, but they also drink moderate amounts of red wine [29]. The health benefits of Mediterranean diets have been attributed, at least in part, to the high consumption of antioxidants provided by fruit, vegetables and wine and to the type of fat, rich in monounsaturated and ω–3 fatty acids from vegetables and fish, and especially to a balanced ω–6/ω–3 fatty acid ratio, as is found in the traditional diet of Greece prior to 1960 [29]. Emerging data reveal that part of the health benefits may be mediated through their nitrite/nitrate content since diets rich in fruits and vegetables, i.e., Mediterranean diet are enriched in nitrite and particularly nitrate [26,27]. In fact, based on a convenience sample from each, a typical Mediterranean diet may contain as much as 20 times higher nitrite and nitrate than a typical western diet [30].

Diet and cancer

In contrast to the evidence on diet and CVD, epidemiological data on the consumption of meats and the risk of cancer sometimes reveal a slightly increased risk. Since dietary factors, which can be numerous and complex, may yield both positive and negative risk associations, they are of great interest to the research community, public health agencies and to the public. A total of 1,479,350 new cancer cases and 562,340 deaths from cancer are projected to occur in the U.S. in 2009 [31]. The U.S. National Institutes of Health estimate overall costs of cancer in 2007 at $219.2 billion. Smoking, poor nutrition, and physical inactivity are important risk factors for cancer. There is evidence that dietary patterns, foods, nutrients, and other dietary constituents are closely associated with the risk for several types of cancer. And while it is not yet possible to provide quantitative estimates of the overall risks, it has been estimated that up to 35 percent of cancer deaths may be related to dietary factors [32]. Many epidemiologic studies have shown that populations that eat diets high in vegetables and fruits and low in animal fat, meat, and/or calories have reduced risk of some of the most common cancers. Coincidently, fruits and vegetables are enriched with nitrite and nitrate from the soil. Colorectal cancer is the third leading cause of cancer-related deaths for both males and females in the U.S. Consumption of specific food components has been associated with risk of colorectal cancer. Dietary factors associated with increased colorectal cancer risk, include red meats and processed meats, while dietary fiber consumption is associated with decreased risk in low risk populations.

The consumption of red meat and, in particular, processed or preserved or cured meats (i.e., meats treated with nitrite as a food additive, including ham, bacon, hot dogs, etc.), has been related to the incidence of colorectal cancer since 1975 in several epidemiological studies. A worldwide recommendation for moderation in the consumption of preserved meats, such as sausages, salami, bacon and ham, was launched by the World Health Organization in 2003 [33]. However, a 2007 report by the World Cancer Research Fund and the American Institute of Cancer Research (WCRF/AICR) has presented a recommendation to "avoid processed meats" [34], based on a meta-analysis of a limited number of selected cohort studies showing increased risk of colorectal cancer with increased intake of processed meats. The summary estimate of relative risk was determined to be 1.21 (95% confidence interval = 1.04–1.42) per 50 g intake/day and was supported by case-control studies. A separate Swedish meta-analysis of 14 cohort studies reported a slightly lower summary hazard ratio estimate of 1.09 (95% confidence interval = 1.05–1.13) per 30 g intake/day [35]. However, these findings must be viewed with skepticism, considering that a relative risk ratio of 1.0 indicates no increase in risk and anything less than 2.0 should not be used for public policy recommendations, according to the U.S. National Cancer Institute [12].

According to the WCRF/AICR summary estimate of relative risk, a decrease of 50 g/day in processed meat consumption may then lower the total number of colorectal cancer cases by approximately 20%. A relative risk estimate of <1.3 would normally receive little attention in the epidemiological community. However, exposure to processed meats is so widespread, even a modest association, if proven causal, may have considerable public health consequences [36]. Curiously, the same literature review by the WCRF/AICR found a statistically significant 26% protective effect against rectal cancer for the highest meat consumption level, an important finding not referenced in the WCRF/AICR report or the press release that accompanied the report. However, there are also large epidemiologic studies showing no association between colorectal cancer and exposure to red or processed meats [37,38], and a recent meta-analysis published since the release of the WCRF/AICR report does not appear to support an independent association between animal fat intake or animal protein intake and colorectal cancer [39]. In addition, the findings of another recent meta-analysis showed no support for an independent relation between red or processed meat intake and kidney cancer [40]. Although some of the summary results were positive, all were weak in magnitude, most were not statistically significant, and associations were attenuated among studies that adjusted for key potential confounding factors, as has often been seen in many cancer epidemiology studies of meat intake.

The International Agency for Research on Cancer (IARC), a United Nations/World Health Organization body headquartered in Lyon, France, has the mission to coordinate and conduct research on the causes of human cancer and the mechanisms of carcinogenesis and to develop scientific strategies for cancer control. The agency is involved in both epidemiological and laboratory research and disseminates scientific information through publications, meetings, courses, and fellowships. IARC Director Peter Boyle and his colleagues responded to the media confusion caused by the release of the WCRF/AICR in an editorial in the *Annals of Oncology* [2]. They strongly objected to the report's downplaying the causative role of tobacco smoking and second-hand smoke in cancer causation and also criticized the report's conclusions on overweight, obesity and diet as major cancer causation factors. Boyle and colleagues specifically cast doubt on the rationale used to classify as "convincing" the evidence linking high meat intake to colorectal cancer risk. In fact, they argued that this conclusion on meat and cancer raised questions about the WCRF/AICR evaluation process and about the robustness of the classification system. Pointing out the fragile grounds on which the conclusions of the WCRF report were based, the editorial's authors felt that the information to the media should have been more cautious and less confusing, and that after decades of dietary research activity, we still do not know how we need to change what we eat to reduce our cancer risk.

Firm evidence of cancer causation in humans is lacking for dietary nitrite and nitrate. A comprehensive review [41] could find no epidemiological evidence linking stomach, brain, esophageal and nasopharyngeal cancers to dietary intake of nitrate, nitrite or *N*-nitroso compounds. This conclusion was further supported by a study showing that cured meat consumption was not linked to adult or childhood brain cancer in the U.S. [42]. The epidemiological data on meats, particularly cured and processed meats, becomes important in terms of exposure because such products are known to contain nitrite and nitrate. There are many other confounding factors in meats (saturated fats for example), but the direct implications for nitrite and nitrate content are far from conclusive. Early reports implicated nitrite and nitrate in processed meats as the culprit. Since the 1950s, when the potential to form carcinogenic *N*-nitrosamines from the reaction of nitrous acid with secondary amines was recognized, the use of nitrite salts as food preservatives has been under intense scrutiny. Numerous case-control studies have been conducted worldwide to determine if there is a link between gastric cancer and nitrate intake [41,43,44].

It is well known that elevated dietary nitrate intake leads to elevated salivary nitrate levels and, after reduction by oral bacteria, higher levels of ingested nitrite [45–47]. Studies in Canada, Italy, Sweden and Germany involving thousands of study subjects have failed to show an association or demonstrated an inverse association between estimated nitrate intake and gastric cancer, perhaps because much of the nitrate was from vegetables [44]. Occupational exposure to very high levels of nitrate occurs in fertilizer workers, who have elevated body burdens of nitrate and elevated

salivary nitrate and nitrite levels show no increased incidence of gastric cancers.[44]. Case-control studies attempting to link nitrate and nitrite consumption to brain, esophageal, and nasopharyngeal cancers have also been inconclusive [41]. In other studies published over two decades, the relationship between the consumption of cured meats during pregnancy and the risk of brain and other tumors in offspring was examined [43]. In a review of 14 epidemiological studies, 13 of which were case-control studies, Blot et al. [43] could not conclude a relationship between cured meat consumption during pregnancy and brain or any other cancers. It may be that in the limited number of epidemiological studies linking nitrate, nitrite or cured meats to a specific cancer site, other as-yet uncharacterized dietary or environmental factors may be involved.

Thus, the concern for the use of added nitrite in processed meats (added nitrate is only used in a very limited number of products outside the U.S.) has waxed and waned as numerous studies were published and independent review and food regulatory panels have been convened to make determinations about nitrite for use as a food additive and public policy. In 2000, the results of a comprehensive battery of rodent carcinogenicity and mutagenicity studies by the U.S. National Toxicology Program (NTP), including a standard, 2-year chronic cancer bioassay of sodium nitrite in rats and mice, were presented to the NTP Technical Reports Review Subcommittee for evaluation. The final NTP Technical Report [48] indicated that the only adverse finding in both rats and mice was an "equivocal evidence" finding that sodium nitrite weakly increased the number of forestomach tumors in female mice but not in male mice or male or female rats. All other organ sites in both rats and mice showed no evidence of carcinogenicity. In short, any suspicion of sodium nitrite's carcinogenicity in rodents was not supported by this state-of-the-art cancer bioassay study. Shortly thereafter, in 2000, nitrite was also reviewed and evaluated for potential listing as a developmental and reproductive toxicant under the Proposition 65 Statute in California. A review of 99 studies on sodium nitrite led the state's Developmental and Reproductive Toxicant Identification Committee of eight independent scientists to conclude that sodium nitrite should not be listed as a developmental toxicant or as a male or female reproductive toxicant under California's Proposition 65 law. The literature and activities associated with the above events were reviewed and published by Archer [49].

The latest review of ingested nitrate and nitrite carcinogenicity was conducted in June 2006 by an expert working group convened by IARC [50]. The IARC working group made a decision to classify nitrate and nitrite for their potential as human carcinogens as follows:

> "Ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans (Group 2A)."

The expert group found that nitrate/nitrite was weakly associated with human stomach cancer only. They also appeared to disregard or misinterpret the findings of the NTP cancer rodent bioassay, which showed sodium nitrite was not carcinogenic in rats and mice. The above conclusion, which in a very narrow sense may be accurate, has in a broader biological sense questionable and minimal practical application. In simple terms, this overall evaluation means that the ingestion of food and water that contain nitrate or nitrite (e.g., spinach and other green leafy vegetables, root vegetables, bread, beer, cured meats), in combination with amines and amides commonly found in food, can react in the stomach to form *N*-nitrosamines and *N*-nitrosamides, which are known animal carcinogens already classified by IARC.

Antimicrobial benefits of nitrite in the food supply

Despite the very weak associations sometimes reported between dietary nitrite/nitrate and cancer, we must not forget the essential nature of these "curative" salts in the safety of the food we eat. The antibotulinal properties of nitrite have long been recognized. The use of nitrite to preserve meat has been employed either indirectly or directly for thousands of years. Nitrite inhibits outgrowth of *Clostridium botulinum* spores in temperature-abused (i.e., non-refrigerated) meat products. The mechanism for this activity was extensively investigated and results from inhibition of iron–sulfur clusters essential to energy metabolism in this obligate anaerobe [51–53]. Importantly, the broad antimicrobial effects of nitrite and implications for human health are still being researched. Commensal bacteria that reside within and on the human body can reduce nitrate, thereby supplying a large and alternative source of nitrite. Thus, ingested nitrite is also derived from reduction of salivary nitrate [54]. About 25% of orally ingested available nitrate is actively secreted into the saliva. This nitrate is partially converted to nitrite by oral bacteria and then disproportionates with formation of NO after entering the acidic environment of the stomach, helping to reduce gastrointestinal tract infection, increase mucous barrier thickness and increase gastric blood flow [54].

Humans, unlike prokaryotes, are thought to lack the enzymatic machinery to reduce nitrate back to nitrite. However, recent discoveries reveal a functional mammalian nitrate reductase [55]. Lundberg and Govoni demonstrated that plasma nitrite increases after consuming nitrate [45]. Therefore, dietary and enzymatic sources of nitrate are potentially large sources of nitrite in the human body. The nitrite in saliva has significant antimicrobial benefits when it is swallowed and converted to nitrous acid and other nitrogen oxides in the intestinal tract. The bactericidal effects of gastric fluids are significantly enhanced by the presence of ingested nitrite. This has been demonstrated for known foodborne pathogens such as *Escherichia coli 0157:H7* and *Salmonella* [56,57]. Nitrite and nitric oxide are also effective bacteriocidal agents against other microorganisms associated with diseases such as *H. pylori*, which has been linked to gastric cancer [58] and skin pathogens [59–61]. As a food additive, nitrite is also important in controlling potential growth of *Listeria monocytogenes* in processed meats. Models that estimate the effects of ingredients on microbial growth show dramatic reductions when nitrite is included [62–68]. The use of such models has enabled formulations of nitrite-cured processed meat products that will not support growth of *L. monocytogenes*. To date, this has not been achieved for uncured counterparts where the only ingredient difference is nitrite. Therefore, addition of nitrite appears essential and requisite for ensuring food safety.

The United States Department of Agriculture (USDA) has done extensive research to develop models to predict growth of pathogens under a variety of conditions [69]. In their models, incorporation of nitrite at current levels significantly inhibits growth of *Listeria, E. coli, Staphylococcus aureus*, and *Bacillus cereus*. By way of illustration, the following predictions were calculated from the USDA Pathogen Modeling program 7.0 model [69] and imply significant pathogen risk reduction when nitrite is included in products (see Table 1).

A second illustration from the same USDA model shown below is in agreement with the reports of enhanced bactericidal effects of nitrite in gastric fluid (see Table 2).

The tradition of curing meats and more recent knowledge surrounding the use of sodium nitrite to address risk of pathogens has been embodied in modern food manufacturing systems. The use of these approaches in cured meats has been estimated to sig-

Table 1
Predicted growth time (hours) for pathogenic organisms from log 3.0 CFU/mL to log 6.0 cfu/mL under anaerobic conditions in broth culture at pH 6.0 in the presence of 2.0% sodium chloride and different levels of sodium nitrite.

Organism	Temperature (°C)	No nitrite	Sodium nitrite (60 ppm)	Sodium nitrite (120 ppm)
Listeria monocytogenes	4	208	283	383
E. coli 0157:H7	9	217	238	284
S. aureus	12	195	230	284
B. cereus	10	144	230	274

Table 2
Predicted 6.0 log reduction time (hours) for pathogenic organisms in broth culture at pH 4.0, 25 °C in the presence of 2.0% sodium chloride and different levels of sodium nitrite.

Organism	No nitrite	Sodium nitrite (60 ppm)	Sodium nitrite (120 ppm)
Listeria monocytogenes	781	158	60
E. coli 0157:H7	147	77	–[a]
S. aureus	1572	780	295
Salmonella	6.3	3.7	2.2

[A] Outside valid parameter range.

nificantly reduce the risk of listeriosis ascribed to such products [70]. If the products were not cured (i.e., no nitrite was used), the risk reduction would be greatly diminished and major efforts would be needed to develop systems to produce equivalently safe products.

Risk–benefit evaluation

Nitrite is now known to be an intrinsic signaling molecule [71,72] capable of producing NO under appropriate conditions as well as forming nitrosothiols [71,73]. Nitrite has been shown to increase regional blood flow [74], increasing oxygen delivery to hypoxic tissues. Enhancing nitrite availability through therapeutic intervention by administering bolus nitrite prior to cardiovascular insult has shown remarkable effects in reducing the injury from myocardial infarction, ischemic liver and kidney injury, stroke and cerebral vasospasm [75–80] in animal models. These first reports on the efficacy of nitrite in cytoprotection have led to nine current clinical trials for the use of nitrite and/or nitrate in both healthy volunteers and patients with specific cardiovascular complications (www.clinicaltrials.gov). Most recently, nitrite has been shown to precondition the myocardium when given 24 h prior to ischemic insult due to the modulation of mitochondrial electron transfer [81] as well as augment ischemia-induced angiogenesis and arteriogenesis [82]. Nitrite also presents remarkable efficacy in promoting regional blood flow in sickle cell patients [83]. Plasma nitrite levels increase in response to exercise in healthy individuals, whereby in aged patients with endothelial dysfunction there is no increase in nitrite from exercise [84]. Nitrite has also been shown to predict exercise capacity [85] and enhance exercise efficiency in humans [86]. Physical activity can even prevent age-related impairment in NO availability in elderly athletes [87]. We now know that nitrite is just as efficacious when given orally at restoring NO biochemistry [88], reversing hypertension from NOS inhibition [89], protecting from myocardial ischemia–reperfusion injury [90], inhibiting microvascular inflammation, reversing endothelial dysfunction and reducing levels of C-reactive protein [91]. This provides proof of concept that dietary sources of nitrite have important physiological functions.

According to the World Health Organization, cardiovascular disease (CVD) is the number one killer of both men and women in the U.S. These deaths represent a staggering 40% of all deaths. Close to 1 million people die each year and more than 6 million are hospitalized. The cost of CVD, in terms of health care and lost productivity, is over $270 billion and increasing as the baby boom population ages. Ischemic heart disease, as the underlying cause of most cases of acute myocardial infarction, congestive heart failure, arrhythmias, and sudden cardiac death, is the leading cause of morbidity and mortality in all industrialized nations. In the United States, ischemic heart disease causes nearly 20% of all deaths (~600,000 deaths each year), with many of these deaths occurring before the patient arrives at the hospital. Heart disease is very likely the result of a dysfunctional endothelium.

One of the most important substances released by our body is NO. The discovery that the human body makes NO from L-arginine revolutionized science and medicine. Continuous generation of NO is essential for the integrity of the cardiovascular system and a decreased production and/or bioavailability of NO is central to the development of cardiovascular disorders. It is also important for communication in our nervous system and a critical molecule which our immune system uses to kill invading pathogens, including bacteria and cancer cell. Reduced NO availability is a hallmark of a number of disorders, including CVD. Understanding strategies to enhance and restore NO homeostasis is critical to developing treatments to cardiovascular disease and more importantly strategies to prevent disease from developing.

The notion that there may be certain foods that can enrich NO within our body is revolutionary. Therapeutics is the branch of medicine concerned with the remedial treatment of disease. It is prudent at this juncture to take a step back and look at nitrite and nitrate as a means of prevention of a number of diseases associated with NO insufficiency. Early intervention to restore NO/nitroso homeostasis through natural dietary means may prove to be a cost-effective and natural means to prevent disease. It is becoming increasingly clear that a deficiency in NO is a hallmark of a number of disease conditions. It is highly unlikely that nature devised a singular pathway which requires a complex five-electron oxidation of a semi-essential amino acid requiring multiple co-factors and prosthetic groups. There is, by design, enormous redundancy in physiology where there are multiple pathways and regulatory processes for critical biological functions. This redundancy ensures that if one pathway is absent or dysfunctional, there is an alternative to produce and regulate critical signaling molecules and pathways. Maintenance of NO homeostasis by nitrite may be the redundant backup system in NO biology. A simple one-electron reduction of nitrite to form NO or bimolecular reaction with thiols to form nitrosothiols is a viable and effective system for recapitulating NO biochemistry. Since at least half of our body's pool of nitrite is derived from what we eat, we can at will, affect NO biochemistry through dietary means. Discovery and recognition of this pathway is likely to affect public health and strategies to prevent and/or treat disease. We are currently faced with an enormous public health crisis as our aging baby boomers succumb to disease, particularly CVD. Treatment and care of our greatest generation is a tremendous economic burden, and developing strategies to prevent disease or reduce injury from a cardiovascular accident will improve patient outcome and enhance quality of life. Recognizing a natural and inexpensive regimen of foods rich in ni-

trite and nitrate to restore NO homeostasis can have profound effects on public health. It is time for health care professionals, clinical nutritionists, dieticians, food scientists and epidemiologists to begin discussion and appreciate contemporary views of nitrite and nitrate in the context of indispensable nutrients.

As with any remedy or treatment, a risk–benefit reward evaluation should be considered. A 2009 Expert Report published by the Institute of Food Technologists (IFT) provided a comprehensive review of the safety and regulatory processes for making decisions about the risks of chemicals in foods [92]. This report described the need for scientists, health professionals and public health authorities to evaluate the health benefits of a specific food or food group when also assessing the health risks that may be posed by the food or food group. Numerous case examples were cited in the report, specifically the weighing of the risks of methyl mercury contamination of certain fish compared to the nutritional benefits of fish consumption, the cancer-reducing effects of coffee consumption even with the presence of trace levels of animal carcinogens in roasted coffee, and the potential cancer risks of heat-induced carcinogens (known to occur at trace levels) in otherwise healthy and nutritional foods and beverages. One of the key conclusions of this expert report was that the risks and benefits of the whole food should be evaluated instead of simply focusing on the risks of individual chemical components of the food.

Using the concepts discussed in the IFT Expert Report, we predict that the benefits of dietary nitrite and nitrate will strongly outweigh any potential risks, particularly for patients with conditions of NO insufficiency. The now recognized and undisputed benefits of dietary nitrite and nitrate should be put in context to the very weak associations to its potential risks. In an editorial comment, Katan [93] eloquently put this point in perspective: "...evidence for adverse effects of dietary nitrate and nitrite is weak, and intakes above the legal limit might well be harmless. This is not unusual in regulatory toxicology. Many chemicals and contaminants might well be safe at intakes above their legal limit. Authorities willingly accept that possibility; erring on the safe side with many chemicals is justified if it keeps just one true carcinogen out of the food supply. But the trade-off changes when excessive caution deprives us of beneficial substances, as claimed by Hord et al. for nitrate [26]. In that case, the evidence for harm needs to be weighed against the potential benefit."

Conclusion

A long history of safe use as a food additive, minimal endogenous production of *N*-nitrosamines and natural metabolism of ingested nitrite all argue that nitrite as currently used in foods is a safe food additive and even beneficial to human health. Dietary intake of nitrate is a well-known marker of a health-promoting fruit and vegetable diet. In addition, nitrite and nitrate per se, as individual chemical compounds, have never been shown to be carcinogens in animal or human studies. Nitrate itself is not capable of reacting with amines to form *N*-nitrosamines. One could ask the question then, how simultaneous ingestion of nitrite/nitrate and nitrosatable amines/amides could be prevented or reduced by public policy considerations stemming from IARC's "probably carcinogenic to humans" conclusion, when the majority of ingested (swallowed) nitrite is endogenously produced in saliva and the major source of nitrate is the consumption of health-promoting fruits and vegetables. Thus, to eat is to ingest nitrite, nitrate, amines and amides, regardless of the specific diet. Specifically, the consumption of processed, cured meats would be no more or less risky given the low amount of residual nitrite in such products ready to consume (approximately 10 ppm in individual servings, and sometimes even undetectable).

There will very likely be considerable debate about the emerging health benefits of dietary nitrite and nitrate in light of the fact that we have been told to limit their intake. However, when considered in the context of evolving research about the biological function of all nitrogen oxides (including nitrite and nitrate) and their metabolism, any changes to current food regulations on nitrite are simply unwarranted, as are any regulatory implications for the unavoidable presence of nitrate in fruits and vegetables. The use of nitrite as a direct food additive represents only a small addition to the body burden of endogenously produced nitrogen oxides. It is hard to believe that the ingestion of nitrite from cured meats or nitrate from fruits and vegetables could have any potential adverse toxic outcomes. As more is understood about the human metabolic nitrogen oxide cycle, it will become apparent that nitrite is a safe and appropriate food additive providing many more benefits to society than risks, and nitrate naturally occurring in fruits and vegetables and some drinking waters poses insignificant risks. In fact, the inherent nitrate content of Traditional Chinese Medicines and their ability to convert nitrite to NO in essence provide over 5000 years of phase 1 safety data in humans with known curative properties for certain ailments and conditions [94].

Consumers should avoid getting caught up in fads to the point where they might ignore sound science and common sense. Dietary fads seem to have varying lifetimes, sometimes 3 months to even 3 years and, often in retrospect, some common sense critical thinking at the early stages of dietary research reporting could avoid a lot of poor dietary advice. We have seen a variety of diet vs. disease fads, some spanning decades, such as the low-fat craze which, according to some observers, has led to significant increases in the incidence of both obesity and type 2 diabetes. Such fads may have negative consequences where they not only fail to improve public health, but actually result in unintended adverse health effects. The importance of dietary variety, balance and moderation should be stressed along with the importance of protective factors (including adequate consumption of fruits and vegetables) in the total diet, combined with a physically active lifestyle. We believe that the weak and inconclusive data on nitrite and nitrate cancer risks described here are far outweighed by the health benefits of restoring NO homeostasis as described by the volumes of published work over the past 10 years [72,95–101]. The risk/benefit balance should be a strong consideration before there are any suggestions for new regulatory or public health guidelines for dietary nitrite and nitrate exposures.

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Journal of Biological Sciences 8 (2): 436-440, 2008
ISSN 1727-3048

Study of Oral Creatine Monohydrate Supplementation on Growth Performance and Histopathological Assessment in Rats and Chickens

[1]Abdolkarim Zamani Moghadam, [4]Habibolla Nazem, [2]Iraj Karimi,
[5]Izad Abolhasani Darani and [3]Hossein Hassanpour
[1]Department of Clinical Sciences,
[2]Department of Pathobiology,
[3]Department of Basic Sciences, Veterinary Medicine College, University of Sharekord, Sharekord, Iran
[4]Department of Basic Sciences, University of Payame Noor (Central), Tehran, Iran
[5]Department of Biology, University of Payame Noor, Esfehan, Iran

Abstract: To clarify the effects of Creatine Monohydrate (CrM) supplementation on growth performance and to evaluate histopathological lesions in rats and broiler chickens. Two species of animals, chickens and rats were maintained on standard conditions. Each of species (thirty six rats and thirty six chickens), were separated into three equal groups of twelve animals. The control group receiving no CrM while the experimental groups received CrM added to the water supply to achieve a dose of 0.25 and 0.5 g kg^{-1} day^{-1}. The body weight and feed intake were measured and feed conversion rate was calculated. All animals were dissected after 15 days; Portions of liver, kidney, skeletal muscle, were separated and prepared for histopathological sections. There were not any significant variations in body weight gain and feed conversion rate between control and creatine-supplemented groups in rats. There were not also any change in feed conversion rate in chickens but body weight gain significantly ($p<0.05$) increased at day 15 of rearing in creatine-supplemented group (0.5 g kg^{-1} day^{-1}) compared to its control. Histopathological changes were not significant in two species. This study showed that creatine do not have any pathological effect on liver, kidney and muscle of rats and chickens and can improve body weight gain in rearing chickens.

Key words: Creatine monohydrate, rat, chicken, growth performance

INTRODUCTION

Creatine is a guanidine compound produced endogenously by the liver and kidneys and is consumed in meat-containing diets (Terjung *et al.*, 2000). It is transported to skeletal muscle, heart, brain and several other tissues by a sodium-dependent transporter (Wyss and Kaddurah-Daouk, 2000). Endogenous synthesis of creatine plus dietary contribution, with half excreted as creatinine (Juhn and Tarnopolsky, 1998a, b). Creatine is an important compound in cellular energy buffering and in the shuttling of energy from the mitochondria to the cytosol. Creatine functions in maintaining cellular ATP homeostasis (Brosnan and Brosnan, 2007). Fatigue during maximal exercise of short duration is partially the result of phosphocreatine (PCr) depletion and inability of phosphocreatine hydrolysis to maintain a high ATP: ADP ratio (Greenhaff, 1997). Thus, nonendurance athletes routinely ingest creatine for a short-term loading period (Toler, 1997). During high-intensity exercise, ATP hydrolysis is initially buffered by PCr via the Creatine Kinase (CK) reaction. Whereas PCr is available instantaneously for ATP regeneration, glycolysis is induced with a delay of a few seconds and stimulation of mitochondrial oxidative phosphorylation is delayed even further. On the other hand, the PCr stores in muscle are limited so that during high-intensity exercise, PCr is depleted within 10 sec. Therefore, if it were possible to increase the muscle stores of PCr and thereby to delay PCr depletion, this might favorably affect muscle performance. Studies have found that dietary supplementation with Creatine Monohydrate (CrM) can increase skeletal muscle (Parise *et al.*, 2001) and brain (Leuzzi *et al.*, 2000) total creatine and phosphocreatine concentrations, with an even greater degree of increase seen in organs with low baseline creatine content such as liver and kidney (Ipsiroglu *et al.*, 2001).

Creatine loading results in increased water retention (Juhn, 1999). Cellular hydration is an anabolic proliferative signal for protein synthesis (Persky and Brazeau, 2001).

Corresponding Author: Hossein Hassanpour, Department of Basic Sciences, Veterinary Medicine College, Sharekord University, Saman St., Sharekord, Iran Tel: +983814424427 Fax: +983814424427

Additionally, Creatine monohydrate increases bone mineral density (Antolic *et al.*, 2007). Thus, creatine supplementation could be expected to increase weight gain and percentage lean.

There has been some concern regarding the potential for CrM toxicity based on two anecdotal human case reports (Koshy *et al.*, 1999; Thorsteinsdottir *et al.*, 2006), one animal study in hypertensive rats (Edmunds *et al.*, 2001) and the fact that carcinogens can be formed if creatine and sugars are heated to high temperatures (Wyss and Kaddurah-Daouk, 2000). In humans, most of the studies that have examined the potential for toxicity have not found evidence of side effects when consumed at recommended doses (Kreider *et al.*, 2003; Mihic *et al.*, 2000; Schilling *et al.*, 2001). Several recent reviews have concluded that dietary CrM supplementation in humans appears to be relatively safe in the short term; however, they cautioned that the long-term side effects have not been evaluated systematically (Juhn and Tarnopolsky, 1998b; Terjung *et al.*, 2000). Encouragingly, more recent studies in humans have not found evidence for CrM-associated toxicity based on blood analysis and side-effect questionnaires in older adults (Brose *et al.*, 2003), young athletes (Terjung *et al.*, 2000)and in patients with neurological diseases (Jan Groeneveld *et al.*, 2003; Walter *et al.*, 2000).

This study was conducted to examine the effects of supplementation of CrM on growth performance and to evaluate its related histopathological lesions in rats and broiler chickens.

MATERIALS AND METHODS

Animals

Chickens: The thirty five -day-old fast -growing broiler chickens (Ross 308) were reared for two weeks in floor pens (density 12 chickens m^{-2}) on shaving saw dust litter. All chickens were kept under standard conditions (temperature, light) and provided *ad libitum* access to water and a standard ration.

Rats: Young Sprague-Dawley rats were used in this study. They were maintained on standard chow and tap water *ad libitum* and were housed under conditions of controlled temperature (25±1°C) and light (07:00-19:00 h). The experimental protocol was designed in compliance with the Principles of Laboratory Animal Care under constitutional rules of Sharekord University.

Each of species (thirty six rats and thirty six chickens), were randomly separated into three equal groups of twelve animals and housed in these groups. The control group receiving no CrM while the experimental groups received CrM (Merck Chemical Co., Bubendorf, FRG)) added to the water supply to achieve a dose of 0.25 and 0.5 g kg^{-1} day^{-1}.

Experiments: Throughout the study, behavior of animals was noted and mortality was recorded daily. The body weight and feed intake were measured daily from each group and feed conversion rate was calculated.

All animals were euthanized using an overdose of Halothane after 15 days of maintenance and were immediately dissected; tissues were visualized and palpated for evidence of gross pathology. Portions of liver, kidney, skeletal muscle (Iliotibialis), were immersed in 10% phosphate-buffered formaldehyde (formalin). These samples were dehydrated in increasing concentrations of ethanol and xylene and embedded in paraffin and 5 μm thick sections were stained with hematoxylin and eosin and cover slipped. The tissue sections were reviewed blindly for histopathological changes.

Statistical analysis: The tissue histopathology was analyzed using the Chi-Square nonparametric statistical test (SPSS-14.0 package). For body weight, feed intake and feed conversion rate as mean±SEM, a one-way ANOVA was employed with Tukey's post hoc test (SPSS-14.0 package). A p-value <0.05 was taken to indicate statistical significance.

RESULTS AND DISCUSSION

It was observed changes in the behavior of experimented rats after day 7. These changes were as hyperactivation and increased aggressiveness but it was not observed any change in behavior of chickens during rearing.

There were not any significant variations in body weight gain and feed conversion rate between control and creatine-supplemented groups in rats (Table 1). There were not also any change in feed conversion rate in chickens but body weight gain significantly ($p<0.05$) increased at day 15 of rearing in creatine-supplemented group (0. 5 kg^{-1} day^{-1}) compared to its control (Table 2). The tissue sections showed nonsignificant histopathological changes such as congestion, hyaline cast, sinusoidal dilatation, proximal tubular cellulitis in the liver or kidney of both species (Fig. 1, 2). No change was observed in muscles.

Table 1: Comparison of mean body weight gain and feed conversion rate in different rat groups

Parameters	Days	n	Control	T_1 (0.25 g kg^{-1} day^{-1})	T_1 (0.25 g kg^{-1} day^{-1})
Body weight gain (g)	3	12	161±10	167±8	156±4
	6	12	167±10	173±8	161±4
	9	12	176±9	178±9	166±5
	12	12	184±10	187±8	168±4
	15	12	191±10	198±9	171±4
Feed conversion rate	3	12	0.02±0.001	0.01±0.002	0.02±0.003
	6	12	0.01±0.002	0.01±0.001	0.02±0.001
	9	12	0.01±0.002	0.01±0.002	0.02±0.001
	12	12	0.01±0.001	0.01±0.001	0.01±0.001
	15	12	0.01±0.001	0.01±0.001	0.01±0.001

Table 2: Comparison of mean body weight gain and feed conversion rate in different broiler groups

Parameters	Days	n	Control	T_1 (0.25 g kg^{-1} day^{-1})	T_2 (0.25 g kg^{-1} day^{-1})
Body weight gain (g)	3	12	1260±41	1217±65	1254±60
	6	12	1550±39	1457±50	1545±54
	9	12	1822±46	1861±91	1769±55
	12	12	2223±90	2113±66	2197±78
	15	12	2349±59	2487±55	2512±55*
Feed conversion rate	3	12	0.09±0.008	0.10±0.007	0.09±0.004
	6	12	0.10±0.006	0.10±0.006	0.10±0.006
	9	12	0.10±0.002	0.10±0.002	0.10±0.002
	12	12	0.09±0.001	0.09±0.003	0.09±0.001
	15	12	0.09±0.003	0.09±0.001	0.09±0.002

**: $p<0.05$ from corresponding control

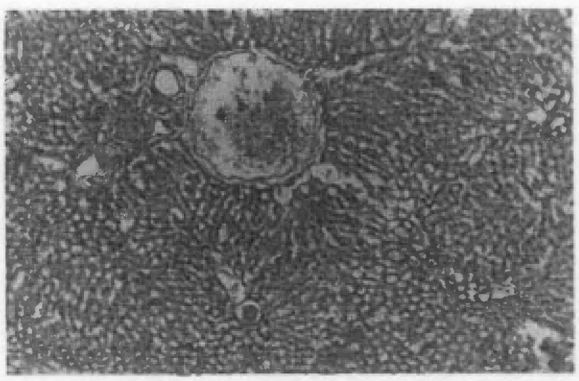

Fig. 1: Liver; Congestion and dilatation of sinusoids (H and E, 4x)

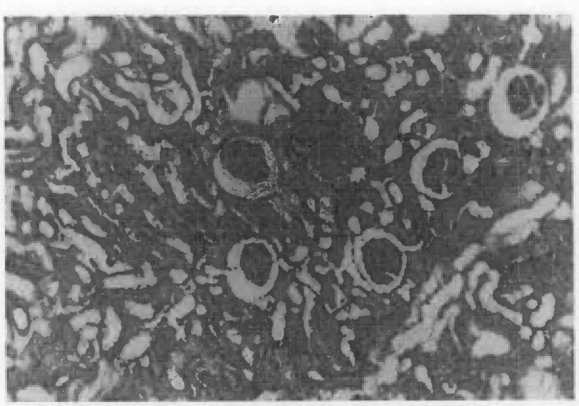

Fig. 2: Kidney; cell swelling and dilatation in renal tubules (H and E, 10x)

Creatine monohydrate is an amino acid derivative that has become a popular sports supplement used to increase muscle performance (Wyss and Kaddurah-Daouk, 2000) in humans. As creatine is taken into the muscle, it is converted to phosphocreatine, which supplies the phosphate needed for the rephosphorylation of ADP, allowing for a delay in the onset of fatigue (Persky and Brazeau, 2001).

Balsom *et al.* (1995) showed that human athletes consuming 20 g of CrM day^{-1} for 6 day increased body mass by 1.1 kg. In the present study, feeding CrM, for 15 days had no effect on body weight gain in rats but had significant elevation in chickens (Table 1). This difference between two species is probably due to differences at the metabolism and function of creatine. It was also observed an aggressiveness in rats that is agreed with the report of Hillbrand *et al.* (1998). They found that there is a positive relationship between Creatine Kinase (CK) and aggressive behavior in 195 males.

At the present study, it was not found any significant histopathological changes in liver and kidney at creatine-supplemented groups of rats and chickens. Juhn and Tarnopolsky (1998b) reported that use of CrM at the fewer than 28 days at recommended does has not been shown to cause significant adverse effects. Poortmans and Francaux (1999) showed that neither short-term, medium-term, nor long-term oral creatine supplements induce detrimental effects on glomerular filtration rate, tubular reabsorption and glomerular membrane permeability. Mayhew *et al.* (2002) determined that oral supplementation with CrM has no long-term

detrimental effects on kidney or liver functions in highly trained college athletes. Cancela *et al.* (2007) reported that 8 week CrM supplementation does not affect on blood and urinary clinical health markers in soccer players. However these data confirm our histopathological results of liver and kidney. We also confirmed that creatine supplementation do not have side effect on muscle mass that is agreed with Bizzarini and Angelis (2004) results. They reported that no strong evidence linking creatine supplementation to deterioration of musculoskeletal functions has been found.

Taken together, the present study showed that creatine supplementation do not have any pathological effect on liver, kidney and muscle of rats and chickens and creatine can improve body weight gain in rearing chickens without any side effect.

ACKNOWLEDGMENTS

We are grateful to Mr. Ahmadi for preparation of pathological tissue sections. This study was supported by Payamenoor University (Esfehan branch) Research Grant.

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ORIGINAL ARTICLE

Taylor&Francis healthsciences

In vitro Studies Indicate that Acid Catalysed Generation of N-Nitrosocompounds from Dietary Nitrate Will be Maximal at the Gastro-oesophageal Junction and Cardia

A. Moriya, J. Grant, C. Mowat, C. Williams, A. Carswell, T. Preston, S. Anderson, K. Iijima & K. E. L. McColl

University Dept. of Medicine and Therapeutics, Western Infirmary, Glasgow, Scotland; Scottish Universities Research and Reactor Centre, Scottish Enterprise Technology Park, East Kilbride, Glasgow, Scotland

Moriya A, Grant J, Mowat C, Williams C, Carswell A, Preston T, Anderson S, Iijima K, McColl KEL. In vitro studies indicate that acid catalysed generation of n-nitrosocompounds from dietary nitrate will be maximal at the gastro-oesophageal junction and cardia. Scand J Gastroenterol 2002;37:253–261.

Background: Dietary nitrate increases saliva nitrite levels and swallowed saliva is the main source of nitrite entering the acidic stomach. In acidic gastric juice, this nitrite can generate potentially carcinogenic N-nitrosocompounds. However, ascorbic acid secreted by the gastric mucosa can prevent nitrosation by converting the nitrite to nitric oxide. **Methods:** To study the potential for N-nitrosocompound formation in a model simulating salivary nitrite entering the acidic stomach and the ability of ascorbic acid to inhibit the process. Concentrations of ascorbic acid, total vitamin C, nitrite, nitrosomorpholine, oxygen and nitric oxide were monitored during the experiments. **Results:** The delivery of nitrite into HCl containing thiocyanate resulted in nitrosation of morpholine, with the rate of nitrosation being greatest at pH 2.5. Under anaerobic conditions, ascorbic acid converted the nitrite to nitric oxide and prevented nitrosation. However, in the presence of dissolved air, the ascorbic acid was ineffective at preventing nitrosation. This was due to the nitric oxide combining with oxygen to reform nitrite and this recycling of nitrite depleting the available ascorbic acid. Further studies indicated that the rate of consumption of ascorbic acid by nitrite added to natural human gastric juice (pH 1.5) was extremely rapid with 200 μmol/l nitrite consumed 500 μmol/l ascorbic acid within 10 s. **Conclusions:** The rapid consumption of ascorbic acid in acidic gastric juice by nitrite in swallowed saliva indicates that the potential for acid nitrosation will be maximal at the GO junction and cardia where nitrite first encounters acidic gastric juice. The high incidence of mutagenesis and neoplasia at this anatomical location may be due to acid nitrosation arising from dietary nitrate.

Key words: Ascorbic acid; Barrett oesophagus; cardia cancer; intestinal metaplasia; nitrate; nitric oxide; nitrite; nitrosoamines; nitrosation; oesophageal cancer; vitamin C

Professor Kenneth E. L. McColl, University Dept. of Medicine and Therapeutics, Western Infirmary, Glasgow, G11 6NT, Scotland, U.K. (fax. +44 141 211 2895, e-mail. K.E.L.McColl@clinmed.gla.ac.uk)

Helicobacter pylori-induced gastritis can explain the majority of cancers of the mid and distal stomach, and although the prevalence of these cancers is falling, along with that of the infection, the prevalence of adenocarcinoma of the proximal stomach and distal oesophagus (collectively referred to as GO junction cancers) is rising (1–4). GO junction cancers differ from cancers of the mid or distal stomach by being associated with normal or high levels of gastric acid secretion rather than with hypochlorhydria or achlorhydria (5–7).

The rising incidence of cancers and precancerous lesions at the GO junction is being attributed to noxious effects of acid, pepsin and possibly bile refluxing from the stomach (8, 9). However, chronic acid/peptic/bile induced mucosal damage may not be sufficient to induce neoplastic change, as exemplified by the lack of association between chronic duodenal ulceration and local cancer occurrence. The mutagen and carcinogen responsible for the rising incidence of lesions at the GO junction remains unknown.

For many years there has been interest in the potential role of endogenously produced N-nitrosocompounds such as nitrosoamines and nitrosoamides in the aetiology of upper GI cancer (10). This is due to the fact that the acidic conditions present in the stomach may allow formation of these N-nitrosocompound through the reaction of nitrite with secondary amines and amides (11). Following the ingestion of nitrate in the diet, large amounts of nitrite are delivered into the acid stomach (12, 13). This is due to the fact that following its absorption in the small intestine 20% of the nitrate is taken up by the salivary glands and secreted into the

mouth (13, 14). Bacteria on the dorsum of the tongue then reduce about 25% of the nitrate to nitrite (15–17). Thus about 5% of all the nitrate ingested in the diet or produced in the body appears in the mouth as nitrite. The salivary glands also actively take up thiocyanate and its concentration in saliva is approximately 1 mmol/l and even higher in smokers (18). The latter anion may also be secreted directly into gastric juice (19). Thiocyanate is a powerful catalyst of the nitrosation of secondary amines by nitrite under acidic conditions (20, 21). The delivery of nitrite and thiocyanate into the acidic environment of the stomach has therefore been regarded as a potentially important source of endogenous formation of carcinogenic nitrosocompounds.

Recently, it has been recognized that the healthy stomach actively secretes ascorbic acid and that its concentration in fasting gastric juice is several times that in plasma (22–25). Ascorbic acid is a powerful inhibitor of the nitrosation reaction. Under acidic conditions, ascorbic acid reduces nitrite or related nitrosating species to nitric oxide and in the process is itself oxidized to dehydroascorbic acid (26–30) (Fig. 1). This removal of salivary nitrite by the ascorbic acid in gastric juice is probably the major mechanism preventing intragastric generation of nitrosocompounds. However, the ability of ascorbic acid to remove nitrite and prevent nitrosation is markedly reduced in the presence of dissolved atmospheric oxygen (31–35). Though ascorbic acid can convert the nitrite to nitric oxide, the latter can combine with oxygen to reform nitrite, which can then react with further ascorbic acid (Fig. 1). This recycling of nitrite in the presence of dissolved oxygen results in consumption of ascorbic acid and then uninhibited nitrosation.

We have recently studied changes in the gastric juice of healthy volunteers following ingestion of 2 mmol nitrate, which is equivalent to that in a typical salad-containing meal (12). Following ingestion of the nitrate, the median salivary nitrite concentration rose from 40 μmol/l to 250 μmol/l within 20 min and remained elevated for >2 h. The increased delivery of nitrite into the stomach in the swallowed saliva was accompanied by a 4-fold fall in the gastric juice concentration of ascorbic acid due to its conversion to dehydroascorbic acid. In most subjects, nitrite was not detectable in the gastric juice before or after the nitrate meal. However, in some of the subjects in whom the increased salivary nitrite caused marked depletion of gastric juice ascorbic acid, nitrite became detectable in the gastric juice. These studies indicated that the increased salivary delivery of nitrite following a nitrate meal could produce nitrosating conditions within the acid secreting stomach through simultaneous depletion of ascorbic acid and consequent accumulation of nitrite.

These observations of the marked depletion of intragastric ascorbic acid following nitrate ingestion stimulated us to re-examine the chemical reactions occurring when nitrite in saliva encounters acidic gastric juice. Our studies indicate that nitrosation within the lumen of the healthy acid secreting stomach is likely to be maximal at the point where salivary nitrite first encounters acidic gastric juice.

Aims

The aim of our studies was to examine the potential for nitrosation to occur under conditions simulating the salivary delivery of nitrite following a nitrate meal into the acid stomach and the ability of ascorbic acid to prevent the process.

Materials and Methods

A model was developed to simulate the interaction of nitrite swallowed in saliva with gastric hydrochloric acid (HCl). A specially designed glass beaker simulating the stomach was filled with 50 ml of HCl and maintained at 37 °C. Sodium thiocyanate at a concentration of 1 mmol/l was added to the HCl to simulate that normally present in human gastric juice (19). Either ascorbic acid (or control infusion alone) was infused continuously into the HCl at a rate of 18 μmol/h to simulate its gastric secretion. Ten minutes after commencing the ascorbic acid or control infusion, nitrite was added to the stomach in a manner simulating its salivary delivery following a salad meal. This was done by injecting a 1 ml bolus of 1.2 mmol/l sodium nitrite every 3 min for 30 min. The secondary amine morpholine was added to the simulated stomach at a concentration of 5 mmol/l to allow monitoring of its nitrosation to nitrosomorpholine. The solutions in the stomach model were constantly mixed with a magnetic stirrer.

Each of the experiments was performed with and without infusion of ascorbic acid and at different gastric pH values i.e. 1.5, 2.5, 3.5 and 5.5. In some experiments, ascorbic acid was infused also at an 8 times higher rate of 150 μmol/h. The different pH values were obtained by using different concentrations of HCL versus NaOH. Each of the experiments was also performed with and without depletion of oxygen from the solutions. For the oxygen depleted experiments, all solutions and chemicals were prepared with boiled distilled water and the apparatus was purged with nitrogen gas for 10 min before commencing the experiment.

Throughout the experiments, samples were taken from the artificial stomach every 20 min for measurement of nitrite, ascorbic acid, total vitamin C and nitrosomorpholine. In some of the experiments the concentrations of nitric oxide and O_2 were also monitored.

Experiments of the rate of reaction of nitrite with ascorbate

Experiments were performed to investigate the rate of the interaction between nitrite and ascorbic acid in hydrochloric acid and human gastric juice.

Twenty millilitres of HCl (pH 1.5), containing ascorbic acid 500 μmol/l and sodium thiocyanate 1 mmol/l, was placed in a small glass vial at 37 °C. A nitric oxide sensor and O_2 probe were immersed in the acid. Two-hundred microlitres of

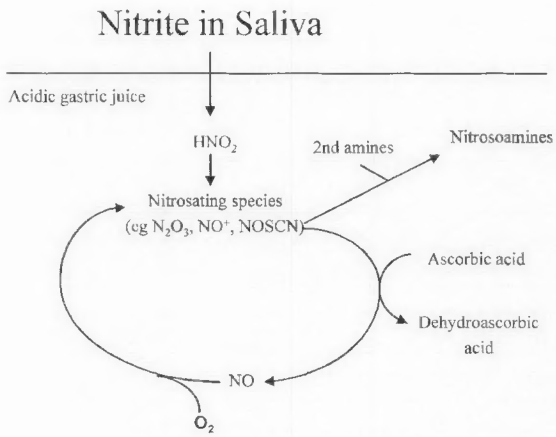

Fig. 1. Diagrammatic representation of chemical reactions occurring when nitrite enters acid solution containing ascorbic acid and 2nd amines. The nitrite forms nitrous acid and nitrosating species that can nitrosate 2nd amines. The ascorbic acid prevents nitrosation by converting the nitrosating species to nitric oxide. However, in the presence of dissolved O_2, the nitric oxide combines with the O_2 to reform nitrous acid and nitrosating species.

20 mmol/l sodium nitrite was injected into the stirred acid, producing a concentration of 200 μmol/l in the container. This concentration of nitrite is equivalent to that likely to occur following a nitrate-containing meal and at the point where saliva first encounters gastric juice (12). The experiments were performed on exposure to air and under oxygen-depleted conditions. Samples were taken after 10, 30, 100, 140 and 300 s of nitrite delivery for measurement of nitrite, ascorbic acid and total vitamin C. The above rate of reaction experiments was repeated with natural human gastric juice obtained from patients undergoing pentagastrin tests of acid secretion. Fresh fasting gastric juice samples were stored at 2 °C immediately after the collection. The sample was divided into two aliquots.

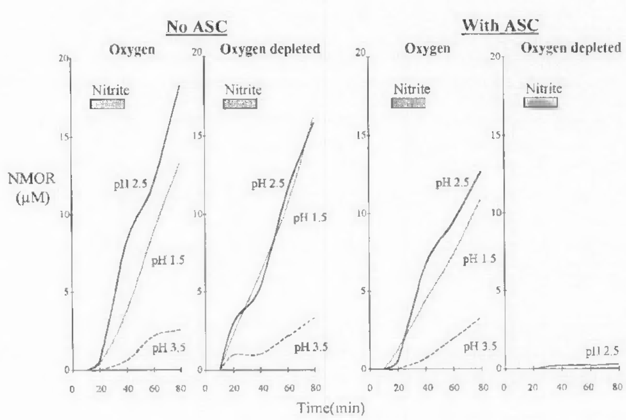

Fig. 2. Rate of formation of nitrosomorpholine (NMOR) when boluses of nitrite are delivered into HCl containing thiocyanate over a 20-min period to simulate salivary delivery following nitrate containing meal. Nitrosation of morpholine was greatest at pH 2.5. Infusion of ascorbic acid (ASC) at rate simulating its gastric secretion was ineffective at preventing nitrosation if there was exposure to atmospheric oxygen.

Ascorbic acid was added to one sample to achieve a concentration of 500 μmol/l. Sodium nitrite was added to the other sample to remove any ascorbic acid. Twenty millilitres of each gastric juice sample was put into small vials at 37 °C and then a bolus of 200 μl of 20 mmol/l sodium nitrite added to the stored solution to produce a final concentration of 200 μmol/l. The concentration of nitric oxide, oxygen, ascorbic acid, total vitamin C and nitrite was monitored as described above.

For the assay of ascorbic acid and total vitamin C in the above rate of reaction experiments metaphosphoric acid and sulfamic acid were prepared from boiled water and collecting tubes were filled with argon gas. This was done in order to minimize any continuing reactions involving oxygen after the samples were obtained. The collecting procedure was accomplished as quickly as possible.

Analyses

Nitrite

Immediately after sampling, the pH of all samples was raised by adding 0.5 ml phosphate buffer to pH 7.25 in order to prevent nitrite being converted to nitric oxide and lost. Samples for nitrite were stored at 4 °C and analysed the same day on 96 well microplates using a modified Griess reaction as previously described (12). Colorometric analysis was performed 15 min after the addition of the Griess reagents using a 540 nm filter.

Ascorbic acid, total vitamin C measurements. A 0.5 ml sample was added to two test tubes. Each contained 0.5 ml equal volumes of 2% metaphosphoric acid/0.5% sulfamic acid and 1 also contained 6 mg/ml dithiothreitol (DTT). The sulfamic acid was added to remove any remaining nitrite. The purpose of the DTT treatment was to regenerate ascorbic acid from any dehydroascorbic acid in the sample, thereby allowing quantification of total vitamin C levels. Upon collection, samples were placed in liquid nitrogen before storage at −80 °C. They were stored for no more than 4 weeks. Ascorbic acid was measured by high performance liquid chromatography as previously described (12) and based upon the method of Sanderson & Schorah (36).

NMOR measurements. A 1 ml solution of 0.08M HCl and 5% w/v sulphamic acid in saturated NaCl was pipetted into screw cap vials. Immediately before the experiments, 100 μl of a 0.01% N-nitroso-di-butylamine (in methanol) internal standard was added to each vial and mixed well. For each time point, 2 ml of sample was withdrawn from the 'stomach' and pipetted into the appropriate vial along with 0.5 ml on extraction solvent mix (45:55 dichloromethane:diethylether) and mixed well to allow transfer of the nitrosoamines into the organic phase. The vials were then left to stand to allow the layers to separate out. The upper (organic solvent) layer was then transferred into a tapered vial and 0.5 ml of fresh solvent added to the original vial and the procedure repeated. The resulting 1 ml of solvent in the tapered vial was then

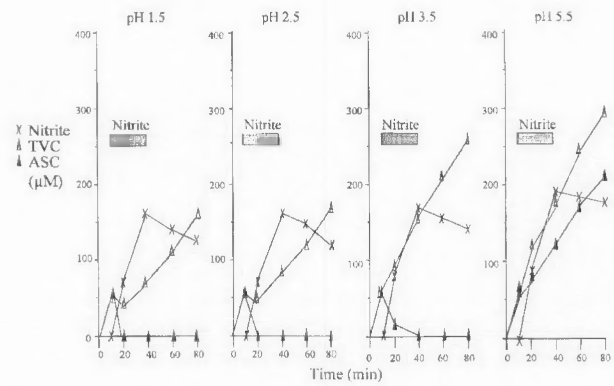

Fig. 3. Addition of boluses of nitrite into HCl containing thiocyanate and into which ascorbic acid (ASC) is being constantly infused. With exposure to atmospheric oxygen, the addition of nitrite causes rapid loss of all the added ascorbic acid at the lower pHs and nitrite accumulates in the stomach.

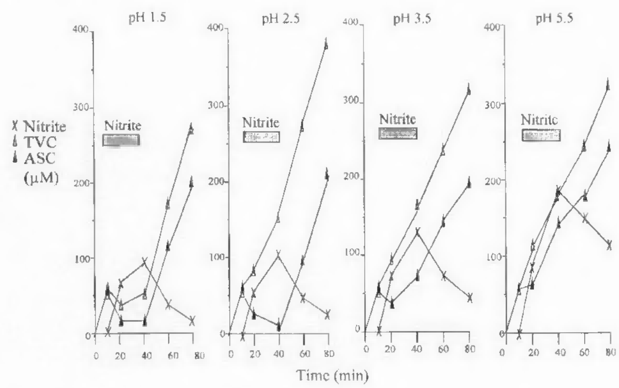

Fig. 4. Experimental design as for Fig. 2, but with depletion of oxygen. Under these conditions, ascorbic acid is always detectable in the stomach and nitrite accumulation at lower pHs is reduced.

concentrated to approximately 50 μl by blowing with a stream of nitrogen gas. These vials were sealed with crimp caps and stored at −20 °C until measurement by GC-MS (gas chromatography–mass spectrometry). All chemicals used were of the highest grade obtainable.

Nitric oxide measurements. The aqueous dissolved nitric oxide concentration was monitored by an isolated dissolved nitric oxide meter (ISO-NO Mark II: World Precision Instruments, Inc. Sarasota, Fla., USA). The nitric oxide electrode was calibrated as follows: 20 ml of 0.1 mol/l H_2S0_4/KI solution was placed in a vial and the nitric oxide sensor placed in it; 200 μl solutions of sodium nitrite were added to produce final concentrations of 20 μmol/l/ 1, 40 μmol/l, 60 μmol/l, 80 μmol/l and 100 μmol/l. Under these conditions the concentration of nitric oxide in the solution equals the concentration of nitrite. The electrode response was linear up to a nitric oxide concentration of 100 μmol/l. The sensitivity of the electrode was 2.00–2.72 nM nitric oxide/pA.

Oxygen measurements. Oxygen tension in the solution was measured by isolated dissolved oxygen meter and electrode (ISO2. World Precision Instruments, Inc. Sarasota, Fla., USA). The oxygen zero point calibration of the sensor probe was done at 37 °C and by bubbling nitrogen gas through the same acid solutions and/or gastric juice as those used in the experiments. The initial O_2 concentration in the experimental solutions was calculated from their temperature and osmolality.

Results

Nitrosation of morpholine by acidified nitrite in the absence of ascorbic acid

We initially studied the nitrosation of morpholine when nitrite was delivered into HCl containing 1 mmol/l thiocyanate but in the absence of any ascorbic acid. Under these conditions, morpholine was nitrosated to nitrosomorpholine and the rate of nitrosation was maximal at pH 2.5 with its concentration reaching 18.3 μmol/l after the 80 min incubation (Fig. 2). The rate of nitrosomorpholine formation was slightly less at pH 1.5 and pH 3.5 and no nitrosomorphine was detected at pH 5.5. The rate of nitrosomorphine formation and the pH optima were unaltered when the experiments were repeated under oxygen-depleted conditions.

Nitrosation of morpholine by acidified nitrite in the presence of ascorbic acid

When the above studies were performed with ascorbic acid being infused into the artificial stomach, the results were profoundly different in the non-oxygen-depleted (Fig. 3) and oxygen-depleted (Fig. 4) conditions.

Non-oxygen depleted system. In the experiments without prior oxygen depletion, the infusion of ascorbic acid at a rate of 18 μmol/h had only a minor effect in reducing the nitrosation of morpholine by the acidified nitrite. The amount of nitrosomorpholine formed was again greatest at pH 2.5, where the concentration at 80 min was 12.7 μmol/l compared to 18.3 μmol/l in the equivalent experiment without ascorbic acid (Fig. 2). In order to understand the inefficacy of the ascorbic acid in preventing nitrosation of morpholine, the concentrations of nitrite, nitric oxide, ascorbic acid and total vitamin C were monitored in the above studies.

The ascorbic acid concentration increased to 59 μmol/l over the first 10 min of its infusion, which was prior to commencing the nitrite delivery (Fig. 3). However, despite continuing to infuse it at the same rate, no ascorbic acid was detected after commencing the nitrite administration in the HCl solutions of pH 1.5, 2.5 or 3.5. However, the total vitamin C concentration did increase, indicating that the ascorbic acid had been oxidized to dehydroascorbic acid. At pH 5.5, the infusion of AA resulted in a progressive rise in its concentration in the HCl with little being oxidized to dehydroascorbic acid following commencement of the nitrite delivery.

Following the addition of the first bolus of nitrite at pH 1.5, there was an immediate rise in nitric oxide concentration,

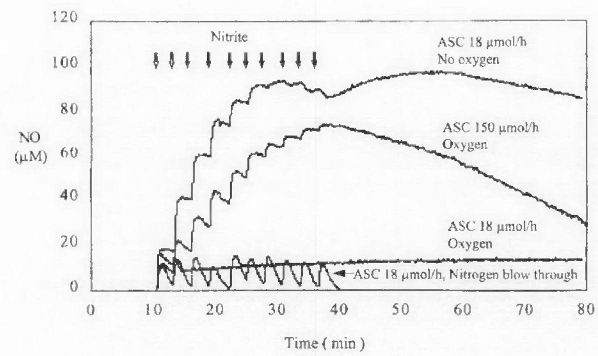

Fig. 5. Formation of nitric oxide (NO) within the artificial stomach when boluses of nitrite are delivered into HCl pH 1.5 containing thiocyanate. Ascorbic acid (ASC) was infused into the stomach throughout the experiment at a rate of 18 μmol/h simulating its gastrin secretion and at the higher rate of 150 μmol/h. The studies were performed in the presence and absence of atmospheric oxygen and also with nitrogen bubbling through the solution.

indicating that all the added nitrite had been reduced to nitric oxide (Fig. 5). However, the addition of subsequent boli of nitrite did not generate nitric oxide but merely increased the nitrite concentration (Figs 3 and 5). The rise in nitrite concentration was apparent at each pH value studied.

Control experiments were performed (in triplicate) to check the stability of ascorbic acid at pH 1.5 with thiocyanate 1 mmol/l at 37 °C without any addition of nitrite. These experiments, performed without oxygen depletion, showed no loss of ascorbic acid over the 80 min period.

The above experiments indicated that the acidified nitrite was rapidly consuming the ascorbic acid and thus preventing the latter from inhibiting the nitrosation of morpholine.

Oxygen-depleted system

When the above studies were repeated using solutions depleted of oxygen, the infusion of ascorbic acid (18 μmol/l) completely prevented nitrosation of morpholine by the nitrite at pH 1.5, 3.5 or 5.5 At pH 2.5, some nitrosomorpholine was detected but only a very low concentration (Fig. 2). The concentrations of nitrite, nitric oxide and ascorbic acid were also markedly different under these anaerobic conditions.

The ascorbic acid concentration fell following the first bolus of nitrite and this fall was most evident at pH 1.5 and 2.5. However, ascorbic acid remained detectable at each time point and, following discontinuation of the nitrite administration, the ascorbic acid concentration progressively rose (Fig. 4).

Nitric oxide concentrations were measured in the pH 1.5 experiment. In contrast to the same experiment performed without oxygen depletion, the nitric oxide concentration increased with each additional bolus of nitrite until reaching the saturation point of the nitric oxide probe (Fig. 5). At the end of the nitrite administration, the nitric oxide concentration

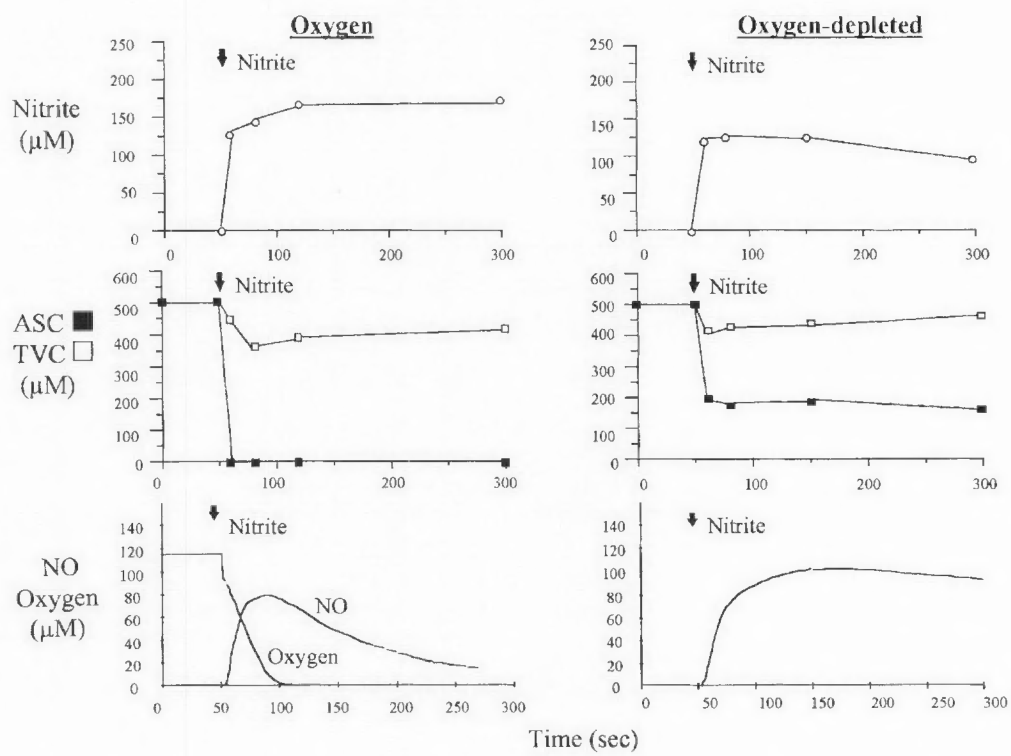

Fig. 6. This shows the rate of oxidation of ascorbic acid (ASC) when a single bolus of nitrite (final concentration = 200 μmol/l) is added to HCl pH 1.5 containing thiocyanate in the presence (left) and absence (right) of atmospheric oxygen. TVC = total vitamin C.

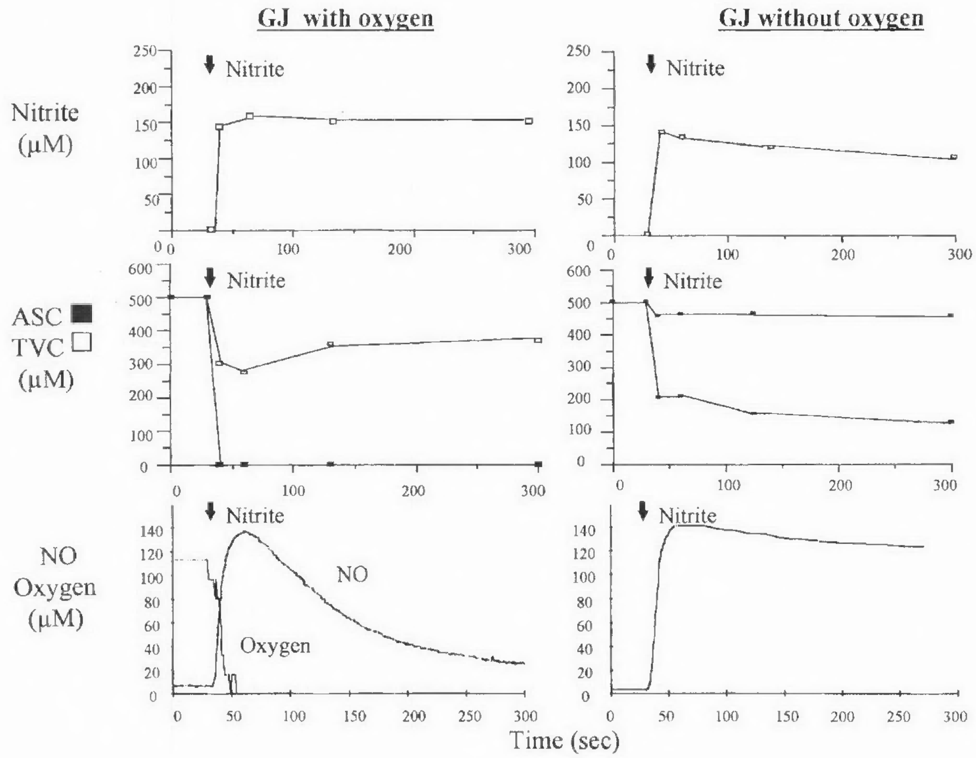

Fig. 7. This shows the rate of oxidation of ascorbic when a single bolus of nitrite (final concentration 200 μmol/l) is added to human gastric juice pH 1.5 in the presence (left) and absence (right) of atmospheric oxygen. TVC = total vitamin C.

was 94 μmol/l and remained at this level for the next 40 min over which it was monitored despite no further addition of nitrite. The nitrite concentrations detected following the nitrite administration were less under these anaerobic conditions and this was most marked at the lower pH levels (Fig. 4).

In the oxygen-depleted system the sum of the measured concentration of nitric oxide (78 μmol/l) and nitrite (54 μmol/l) at 10 min after starting the nitrite infusion was greater than the concentration that could be accounted for from the quantity of nitrite added (86 μmol/l). In addition, the very rapid rise in the nitric oxide concentration following the nitrite addition indicated it was all rapidly converted to nitric oxide. It was assumed that some or all of the nitrite detected by the Griess reaction was probably an artefact formed after collection of the sample as a result of the nitric oxide within the solution reacting with O_2 in the pH 7 buffer solution to form nitrite. In order to confirm this, the experiment was repeated with N_2 constantly bubbling through the artificial stomach to remove any nitric oxide formed. Under these conditions no nitrite was detected.

Ability of high doses of ascorbic acid to inhibit acid nitrosation in the presence of atmospheric oxygen

Further studies were performed at pH 1.5 without oxygen depletion in which the ascorbic acid was infused at the 8-fold higher rate of 150 μmol/h. This increased rate of ascorbic acid infusion completely prevented the nitrosation of morpholine. This much higher infusion rate of ascorbic acid also produced much higher concentrations of nitric oxide, concentrations that were equivalent to those seen in the lower (physiological) ascorbic acid concentration experiment with oxygen depletion (Fig. 5). Ascorbic acid was present at considerable concentration throughout the experiment with only approximately 35%–40% being oxidized to dehydroascorbic acid after commencing the nitrite administration.

In this high-dose ascorbic acid experiment without O_2 depletion, it was possible to calculate the molar loss of ascorbic acid relative to nitrite added. This indicated that at the end of the nitrite administration the amount of ascorbic acid consumed was 5.9 times greater than the amount of nitrite added and at 40 min after completion of the nitrite administration the ratio was 7.9. This represented 12 and 16 times the amount of ascorbic acid predicted by the stoichiometry of 1 molecule of ascorbic acid converting 2 molecules of nitrite to nitric oxide.

Rate of nitrite-ascorbic acid reaction

The rate of the interaction of nitrite and ascorbic acid in HCl was examined by adding a bolus of nitrite (to produce a final concentration 200 μmol/l) to HCl pH 1.5 containing thiocyanate 1 mmol/l and with and without exposure to atmospheric air. The nitric oxide concentration rose from 0

to 50 μmol/l within 10 s of adding the nitrite (Fig. 6). There was also rapid disappearance of ascorbic acid due to its oxidation to dehydroascorbic acid by the nitrite. The rate and amount of consumption of ascorbic acid was greater in the experiments exposed to atmospheric O_2 with all the ascorbic acid being oxidized within 10 s of adding the nitrite. In the experiments exposed to atmospheric O_2 the addition of nitrite was also accompanied by a rapid fall in O_2 concentration (Fig. 6). All of the dissolved O_2 (120 μmol/l) was consumed within 50 s of adding the nitrite.

Identical results were obtained when the studies described immediately above were repeated with natural human gastric juice pH 1.5 containing 500 μmol/l ascorbic acid and with no additional thiocyanate added (Fig. 7).

Discussion

Our studies simulated chemical reactions occurring when nitrite swallowed in saliva meets gastric HCl. The rate of nitrite delivery was based on our recent human study following ingestion of 2 mmol nitrate, which is equivalent to that contained in a standard salad meal (12). The rate of delivery of ascorbic acid into the simulated stomach was based on the rate of gastric secretion of the vitamin by the healthy stomach (22–25). The concentration of thiocyanate was based on that reported in human gastric juice (19).

In our initial in vitro experiments, we studied the effect of delivering nitrite into the artificial stomach in the absence of ascorbic acid. The stomach contained physiological concentrations of thiocyanate (1 mmol) and morpholine as a potentially nitrosatable secondary amine. Under such conditions, the morpholine was nitrosated and the rate of nitrosomorpholine formation was maximal at pH 2.5. The rate of nitrosomorpholine formation was unaffected by the depletion of oxygen from the solutions.

Chemical nitrosation occurs when nitrosating species such as N_2O_3, NO^+ or NOSCN are formed from nitrous acid and react with amines which are in their unprotonated state (11, 37) (Fig. 1). The proportion of nitrite present as nitrous acid is greatest at lowest pH (pKa nitrous acid = 3.3). However, the proportion of morpholine in its unprotonated state decreases with falling pH (pKa morpholine = 8.3). Consequently, conditions are optimal for nitrosation at a pH value of approximately 3.5. Thiocyanate which is secreted in saliva and present in gastric juice is a powerful catalyst of the nitrosation reaction. It also lowers the optimal pH for nitrosation to 2.5 (20, 21, 38).

We proceeded to assess the ability of ascorbic acid to prevent the nitrosation reaction by infusing it into the stomach at a rate equivalent to its gastric secretion. Ascorbic acid effectively competes with secondary amines for the nitrosating species, reducing the latter to nitric oxide and in the process the ascorbic acid is oxidized to dehydroascorbic acid (26–29) (Fig. 1). Under our experimental conditions, the ascorbic acid was ineffective at preventing nitrosation of morpholine despite being administered at four times the molar amount stoichemically required for converting nitrite to nitric oxide. In addition, at the pH values at which nitrosation occurs (1.5–3.5) no ascorbic acid was detected after commencing the nitrite administration, having all been oxidized to dehydroascorbic acid or other inactive metabolites.

Previous studies have observed that dissolved O_2 reduces the efficacy of ascorbic acid to remove nitrite and prevent nitrosation under acid conditions (31–35). When we repeated our studies under anaerobic conditions it resulted in ascorbic acid being detectable throughout the experiment, much greater loss of nitrite, much higher concentration of nitric oxide and prevention of nitrosomorpholine formation. We further investigated the extent to which dissolved oxygen was affecting the consumption of ascorbic acid by nitrite under acidic conditions. In order to do this, we infused ascorbic acid at a much greater rate at pH 1.5 without oxygen depletion and calculated the ratio of the amount of ascorbic acid consumed to nitrite administered. Under such conditions, 16 times the amount of ascorbic acid was consumed by the nitrite than expected from the stoichiometry.

The increased consumption of ascorbic acid by nitrous acid in the presence of O_2 is thought to be due to recycling of the nitric oxide (31–35) (Fig. 1). The nitric oxide formed from the reaction of nitrous acid with ascorbic acid combines with oxygen to reform nitrous acid and this recycling will continue, provided adequate oxygen is available, until all the ascorbic acid is oxidized (Fig. 1). The rate of this recycling and consequent consumption of ascorbic acid will depend highly upon the initial concentration of nitrite added and consequent concentration of nitric oxide initially generated. This is due to the fact that the rate of reaction of nitric oxide with O_2 to form nitrite is related to the square of the nitric oxide concentration × O_2 concentration.

We extended previous work in this area by examining the rate of the chemical reactions occurring when nitrite in saliva first encounters acidic gastric juice containing ascorbic acid and thiocyanate and exposed to atmospheric oxygen. For this we employed a nitric oxide probe allowing real-time measurement of the rate of appearance of nitric oxide, the main product of the reaction of nitrite with ascorbic acid. We also used an oxygen probe allowing real-time measurement of the rate of oxidation of the nitric oxide back to nitrite. The rate of appearance/disappearance of the other relevant chemicals, nitrite, ascorbic acid and dehydroascorbic acid was also assessed. The above studies demonstrated that the reaction of nitrite with ascorbic acid in acidic gastric juice of pH 1.5 is extremely rapid. The great majority of the nitrite is converted to nitric oxide within 10 s of encountering the gastric juice. Likewise, the ascorbic acid concentration fell from 500 μmol/l to 0 μmol/l within 10 s. This profound and rapid depletion of ascorbic acid in the presence of O_2 indicates very rapid recycling of nitric oxide back to nitrite. The recycling was apparent in the rate of depletion of O_2 with its concentration falling from 120 μmol/l to 0 μmol/l within 20 s of adding the nitrite.

In summary, we have studied the chemical reactions that occur when nitrite in saliva encounters acidic gastric juice containing ascorbic acid and the potential for nitrosation to occur. We have confirmed that dissolved O_2 markedly impairs the ability of the ascorbic acid to inhibit acid nitrosation. This is explained by the nitric oxide, formed by the reaction of ascorbic acid with nitrous acid, combining with the O_2 to reform nitrous acid and this recycling continuing until either the ascorbic acid or the O_2 as depleted. We have also extended previous work by studying the rate and extent to which these chemical reactions occur when nitrite in saliva first encounters acidic gastric juice containing ascorbic acid. These indicate that in gastric juice of pH 1.5, the reactions are extremely rapid, with most of the nitrite being converted to nitric oxide and ascorbic acid consumed within 10s. In addition, in the presence of dissolved O_2 and thiocyanate, the nitrite can consume several times its equivalent molar concentration of ascorbic acid within 10 s due to the rapidity of the recycling phenomenon.

It has been considered previously that the stomach represents a single homogenous compartment with respect to acid-mediated luminal nitrosation. However, our present studies indicate that there is likely to be a nitrosative gradient within the healthy acid secreting stomach with the intraluminal nitrosative potential being maximal at the site where the saliva delivering the nitrite first encounters acidic gastric juice. This will correspond anatomically to the GO junction and cardia. Gastric luminal nitrosation is likely to be maximal at this location for several reasons: (1) Acid nitrosation depends upon the ratio of nitrite delivery to ascorbic acid delivery. Nitrite is delivered into the acid stomach in saliva, whereas the ascorbic acid is secreted by the gastric mucosa. The ratio of nitrite to ascorbic acid will therefore be highest at the point where saliva first encounters gastric juice. (2) The very rapid rate of reaction between nitrite and ascorbic acid allows rapid depletion of ascorbic acid at the point where the reactants first meet. (3) The role of oxygen in enhancing the consumption of ascorbic acid by nitrous acid will also increase the potential for acid nitrosation at the point where saliva first encounters acidic gastric juice. Swallowing is the major source of delivering atmospheric air and thus oxygen into the stomach and the oxygen in saliva (31–41) will increase the local consumption of ascorbic acid.

The GO junction and cardia is also the region of the stomach with the optimal postprandial pH for acid nitrosation. Following a meal, the buffering effect of the food raises the intragastric pH. However, we have recently demonstrated that the GO junction and cardia escape the buffering effect of food and maintain a pH of 2.5 (42). The GO junction and cardia thus have the optimal pH for acid nitrosation during the postprandial period when salivary nitrite delivery is maximal.

The anatomical site where acid nitrosation will be maximal corresponds to the site of highest incidence of intestinal metaplasia and adenocarcinoma in the healthy acid-secreting stomach. This raises the possibility that the high incidence of mutagenesis and carcinogenesis at the GO junction and cardia may be the consequence of local luminal production of nitrosocompounds. Such compounds are lipophylic and will rapidly diffuse into the local epithelium. In addition, they are known to produce intestinal metaplasia and carcinoma of the oesophagus and stomach in laboratory animals (43–45).

Intragastric formation of nitrosocompounds has been incriminated in the carcinogenises of cancer of the mid and distal stomach (46). Such patients are achlorhydric or hypochlorhydric, leading to accumulation of nitrite in the gastric lumen and colonization of the stomach with bacteria able to convert the nitrite to nitrosocompounds. In the acid-secreting stomach, this bacterial mediated nitrosation will not occur. However, acid-mediated nitrosation due to acidification of salivary nitrite may occur and our studies indicate that this is likely to be maximal at the GO junction, which is the site of adenocarcinoma in such patients.

A role for acid catalysed nitrosation in the aetiology of GO junction cancer could explain the marked rise in incidence of the cancer over the past 20 years (1–4). Dietary nitrate is the main source of salivary nitrite (12–14). Human exposure to dietary nitrate in the Western world markedly increased between 1950 and 1980 due to the huge increase in use of nitrogenous fertilisers (47, 48). The increase in incidence of GO junction cancer is thus occurring at a time consistent with carcinogenic consequences of increased dietary nitrate exposure.

Acknowledgement

Grant support from the Japanese Society of Clinical Pharmacology is gratefully acknowledged.

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Received 18 June 2001
Accepted 4 September 2001

Z Lebensm Unters Forsch A (1998) 207:477–480

ORIGINAL PAPER

M. Murkovic · D. Steinberger · W. Pfannhauser

Antioxidant spices reduce the formation of heterocyclic amines in fried meat

Received: 23 April 1998

Abstract Heterocyclic aromatic amines (HAs) are mutagenic compounds that are formed during heating of meat and fish. These substances are reaction products of creatine with amino acids and carbohydrates. It is recommended that exposure to these probable human carcinogens should be minimised. Five heterocyclic aromatic amines which occur in beef were investigated: 2-amino-3-methyl-imidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethyl-imidazo[4,5-f]quinoxaline (4,8-DiMeIQx), and 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP). Clean-up was done by acid-base partition followed by SPE using blue cotton. HPLC analysis was carried out by using electrochemical detection for IQ- and IQx-type compounds and fluorescence detection for PhIP. The concentrations of the aromatic amines were as follows: IQ, 10.2 μg/kg; MeIQ, 2.46 μg/kg; MeIQx, 13.2 μg/kg; 4,8-DiMeIQx, 2.26 μg/kg; and PhIP, 5.48 μg/kg. The application of spices (rosemary, thyme sage, garlic, brine) reduced the content of the HAs below 60% of the amount found in the control.

Key words Heterocyclic amines · Fried meat · Spices

Introduction

The major intrinsic mutagenic compounds in food isolated to date from heated meat and fish products are heterocyclic amines (HAs). It has been concluded from epidemiological studies that these compounds are probably responsible for the elevated risk of colon and other types of cancer caused by the consumption of fried meat products [1, 2]. The International Agency on Cancer Research has classified several of the HAs as possible, and 2-amino-3-methyl-imidazo[4,5-f]quinoline (IQ) as a probable, human carcinogen and has recommended a reduction in exposure to these compounds [3]. However, in order to estimate and minimize exposure to HAs, it is necessary to analyze their quantities in foods.

HAs are normally found in heated meat and meat products. The concentration of HAs depends on the time and temperature regime used during cooking [4, 5]. An increase in the cooking temperature normally results in an increased mutagenic activity up to 200–250 °C. If the surface temperature is increased to 300 °C the mutagenic activity increases markedly. At these temperatures other types of heterocyclic compounds are formed due to pyrolysis of amino acids [4]. The temperature profile which is normally found is described by Skog [4] with surface temperatures of 140–200 °C and a maximum temperature within the piece of meat of 100 °C. When, for example, poultry is fried at a surface temperature of not higher than 140 °C, the content of HAs is comparably low [6]. The temperature gradient is formed mainly within the crust. The HAs are usually formed as a product of the Maillard reaction. It was suggested by Jägerstad et al. [7] that the IQ compounds can be produced via the Maillard reaction from creatine, free amino acids and hexoses that are present in foods of animal origin. The formation of several imidazoquinolines and imidazoquinoxalines from these precursors has also been demonstrated to occur following the heating of model systems with creatine or creatinine, amino acids and sugars in diethylene glycol [8]. The substances that are mainly found in cooked meat and fish products are: (IQ) [9]; 2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline (MeIQ) [10]; 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline (MeIQx) [11]; 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx) [12]; and 2-amino-1-methyl-6-phenyl-imidazo[4,5- b]pyridine (PhIP) [13] (Fig. 1).

M. Murkovic (✉) · D. Steinberger · W. Pfannhauser
Graz University of Technology, Department of Biochemistry and Food Chemistry, Petersgasse 12/2, A-8010 Graz, Austria
Tel.: +43-316-8736495, Fax: +43-316-8736971

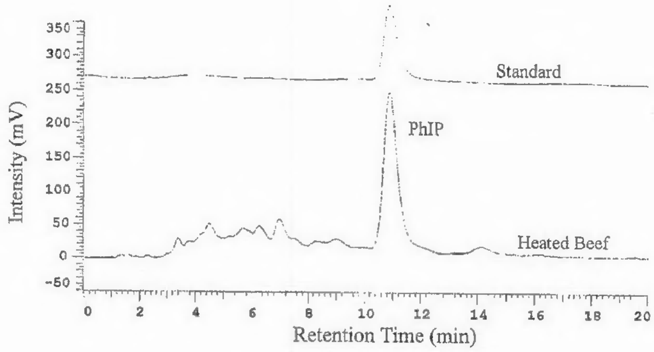

Fig. 1 HPLC analysis of 2-amino-1-methyl-6-phenyl-imadazo [4,5-b]pyridine (*PhIP*) in fried beef using fluorescence detection

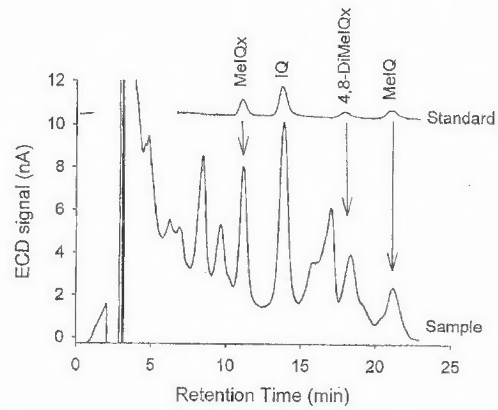

Fig. 2 HPLC analysis of heterocyclic amines (HAs) in fried beef using electrochemical detetion. *MeIQx* 2-Amino-3,8-dimethyl-imidazo[4,5-fquinoxaline; *IQ* 2-amino-3-methyl-imidazo[4,5-f]-quinoline; *4,8-DiMeIQx* 2-amino-3,4,8-trimethyl-imidazo[4,5-f]-quinoxaline; *MeIQ* 2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline

The analysis of HAs is normally carried out by acid-base partition followed by two different SPE procedures. An overview concerning the analysis of HAs is given in [14]. Blue cotton which was used for this study is a trisulfo-copper-phthalocyanin complex bound to cotton. It was introduced for the clean-up of HAs by Hayatsu et al. [15]. Blue cotton has a high affinity for condensed planar aromatic structures and especially HAs.

The influence of antioxidants on the formation of heterocyclic amines was especially shown in model systems. First experiments revealed an increased mutagenic activity when a model system with amino acids, creatine and carbohydrates were heated in the presence of hydroquinone as pro-oxidant [16]. Addition of antioxidants to the reaction mixture also increased the formation of MeIQx [17], whereas flavones showed an inhibitory effect on the formation of HAs [18]. Several spices are known for their antioxidant activity. The active constituents of these are, for example, rosmanol, rosmadial, carnosol, carnosic acid, epirosmanol and methyl camosate in sage or rosemary [19–21]. The antioxidative principles in garlic are known to be S-alkenyl cysteine sulphoxides [22], and many other sulphur components also account for the antioxidant activity of the essential oil of garlic [23].

The aim of this study was to investigate the influence of spices that are known to have antioxidative properties on the formation of HAs. Since radical reactions play an important role in HA formation, it was hypothesized that antioxidants should reduce the content of these mutagenic substances in fried meat.

Materials and methods

Frying of beef. A piece of beef (bos taurus, *M. glutaeus medius*) was fried for 20 min on both sides simultaneously using a teflon-coated heating device. The size of the meat was 10 cm × 15 cm with a thickness of 1 cm. No salt or oil was added. The temperature was recorded by use of PT100 sensors and a data logger (Grant Instruments, Cambridge, UK). The temperature profile is shown in Fig. 2. Addition of spices was done by spreading the ground powder on the surface of the meat 24 h prior to heating. The spices used were rosemary (*Rosmarinus officinalis*), sage (*Salivus officinalis*), thyme (*Thymus* spp.), and garlic (*Allium sativum*). Brine, which is normally used for curing of meat, was applied in the same manner since it is known that nitrite has antioxidative properties.

Chemicals. All chemicals used were of analytical grade. Water was distilled twice and additionally purified with activated charcoal. All solvents, e.g. methanol and dichloromethane (DCM), were of HPLC grade and were purchased from Merck (Darmstadt, Germany). Synthetic HAs used as reference materials were obtained from Toronto Research Chemicals (Toronto, Canada). Blue cotton was made in our laboratory according to Hayatsu et al. [15]. Remazol Turquoise Blue G 133 which is a trisulpho-copper-phthalocyanine complex used for preparation of blue cotton was a gift from Hoechst (Vienna, Austria). The copper complex was coupled to cotton. Dried spices were used and were bought in the local market.

Sample Preparation. The fried meat was homogenized with a mixer (B400, Büchi, Flawil, Switzerland) and freeze-dried. Freeze drying was done so that the samples could be stored before clean-up without any risk of further reactions in the meat. About 10 g of the homogenized grilled beef was suspended in 25 ml of 1 *N* NaOH and sonicated at room temperature for 15 min. The slurry was applied to a column filled with Extrelut (Merck, Darmstadt, Germany) and extracted slowly 5 times with 40 ml of DCM. The DCM was directly applied to a blue cotton column (10 ml). The HAs were eluted from the blue cotton with 100 ml methanol/ammonia (50/1). After evaporation of the eluent the residue was dissolved in 1 ml methanol and diluted with 3 ml of water. This solution was applied to a second solid phase extraction using a C18 cartridge (SepPak, Waters, USA). Elution was done with 6 ml of MeOH/water (70/30).

HPLC analysis. The extracts were evaporated to dryness and finally dissolved in 1 ml methanol. Then 20 μl of the sample was injected into a HP 1100 HPLC with an electrochemical detector (HP 1049A) and for fluorescence detection into a Merck/Hitachi (LaChrom) liquid chromatograph with a fluorescence detector (L 7480), both equipped with a LiChrospher 60 RP-select B (5 μm, 250 × 4 mm) and a precolumn LiChroCART 4-4 (LiChrospher 60 RP18 endcapped, 5 μm). The columns were eluted with a mobile phase of methanol/acetonitrile/acetic acid/water (80/140/20/760) at a pH of 5.1. The flow rate was 1.0 ml/min and the effluent was monitored at +0.85 V for determination of the IQ-type sub-

stances. Fluorescence detection was carried out using 0.01 M triethylamine in water adjusted with phosphoric acid to pH 6.5 (50%) and methanol (50%) as eluent monitoring at $\lambda_{ex}=316$ nm, $\lambda_{em}=370$ nm for determination of PhIP. Separations were carried out at ambient temperature.

Identification and quantification. The HAs were identified by comparing their retention times with those of authentic standards. Additionally, some samples were analyzed by diode array detection and the spectra were compared with those of authentic standards. The quantification was based on a standard addition method which was applied for every substance. All standard solutions were prepared daily from a stock solution which was stored in the dark at −20 °C. The experiment was repeated 3 times using the same heating procedure. Standard addition was carried out with three concentration levels for all five compounds. The SD was calculated using linear regression of the standard addition. The standard addition was used to calculate the recovery for all substances in every experiment.

Results and discussion

During frying of the beef for 20 min at temperatures of up to 180 °C, HAs were formed. The content of the HAs (13.2 μg/kg MeIQx; 10.2 μg/kg IQ; 2.26 μg/kg 4,8-DiMeIQx; 2.46 μg/kg MeIQ and 5.28 μg/kg PhIP) was comparable to other published results [13, 24–28]. The cooking conditions used were comparable to those used for household cooking and gave an attractive, well-cooked piece of meat.

Table 1 lists the recovery and reproducibility of the analysis of HAs in beef. The calculation of the recovery was done by using the standard addition method. The recovery was around 70% for all HAs, with a lower recovery for 4,8-DiMeIQx of 61% and IQ with a higher recovery of 77% using this clean-up method. Figure 1 shows the HPLC analysis of PhIP using the very sensitive and selective fluorescence detection. The chromatogram obtained by electrochemical detection for MeIQx, IQ, 4,8-DiMeIQx and MeIQ is shown in Fig. 2.

In Fig. 3 is can be seen that a higher temperature and a longer heating time resulted in a significant increase in the HA content. Especially the level of MeIQx increased dramatically at higher temperatures. The level of PhIP did not increase that much, which can be explained by the lower stability of PhIP at higher temperatures and therefore faster degradation under these cooking conditions.

Table 1 Recovery of heterocyclic amines (HAs) in beef and reproducibility of the analysis. *MeIQx* 2-Amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline; *IQ* 2-amino-3-methyl-imidazo[4,5-f]quinoline; *4,8-DiMeIQx* 2-amino-3,4,8-trimethyl-imidazo[4,5-f]quinoxaline; *MeIQ* 2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline; *PhIP* 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine

	MeIQX	IQ	4,8-DiMeIQx	MeIQ	PhIP
Mean (μg/kg)	4.3	3.7	4.0	1.3	4.1
Recovery (%)	70	77	61	69	69
SD	0.19	0.17	0.39	0.32	0.13
SD (%)	4.5	4.4	9.7	24	3.2

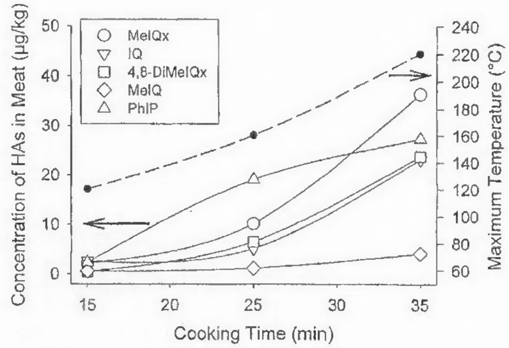

Fig. 3 Influence of cooking time and temperature on the formation of HAs in beef. For abbreviations, see Figs. 1 and 2

Table 2 Influence of antioxidant spices on the formation of HAs in heated beef. For abbreviations, see Table 1

Without	Content of HAs in spiced meat compared to standard cooking procedure (%)				
	MeIQx	IQ	MeIQ	PhIP	4,8-DiMeIQx
Spices	100	100	100	100	100
Rosemary	62	31	36	25	61
Thyme	39	26	39	25	0
Sage	60	0	23	0	36
Garlic	29	68	60	46	22
Brine	15	14	51	34	0

The application of spices to the surface of the meat resulted in significantly lower amounts of HAs. Table 2 also shows that the spectrum of HAs formed differed when different spices were used. Brine, which contains sodium nitrite, also showed antioxidative properties that led to a reduction in the HA content.

Addition of dried spices to the surface of the meat prior to heating resulted in a significant reduction in the content of HAs. This effect can be explained by the antioxidative properties of the spices. Radicals play an important role during the formation of nitrogen heterocyclic compounds in the Maillard reaction. The reactive intermediates (e.g. pyrazinium cation radicals [29] and pyridinium cation radicals [30]) could also be intermediates in the formation of HAs. Antioxidants can inactivate these radicals and therefore reduce the formation of HAs in meat during heating. In contrast to the antioxidative effect of the spices tested, pro-oxidant additives like Fe^{2+} ions in the form of myoglobin increased the HA content (Table 2). The pro-oxidative properties have been described in detail by Halliwell and Gutteridge [31]. Myoglobin, a complex containing Fe^{2+} which is known for its pro-oxidant properties [32], was also tested for its effect on HA formation. Applied on the surface in the same manner as the spices the formation

Table 3 Effect of myoglobin added to the surface of the meat prior to frying. For abbreviations, see Table 1

	HA content using standard procedure (μg/kg)	HA content with addition of myoglobin (μg/kg)
MeIQx	13.2	21.9
IQ	10.2	16.2
4,8-DiMeIQx	2.26	14.7
MeIQ	2.46	1.06
PhIP	5.48	10.4

of the HAs was increased significantly. The content of the HAs after treatment with myoglobin is shown in Table 3.

The data showed a significant increase of all tested HAs except MeIQ of at least 50%. Especially noteworthy, 4,8-DiMeIQx increased 6 times when myoglobin was added to the meat. MeIQ decreased from 2.46 μg/kg to 1.06 μg/kg.

The results show the positive effect of spices that are normally used during household preparation of meat on the formation of carcinogenic HAs. The method described may offer a useful means of reducing the formation of carcinogens in cooked foods and of minimizing the hazards to human health.

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